



**QUEEN'S  
UNIVERSITY  
BELFAST**

## **Role of capsular modified heptose in the virulence of *Campylobacter jejuni***

Wong, A., Lange, D., Houle, S., Arbatsky, N. P., Valvano, M. A., Knirel, Y. A., Dozois, C. M., & Creuzenet, C. (2015). Role of capsular modified heptose in the virulence of *Campylobacter jejuni*. *Molecular Microbiology*, 96(6), 1136-1158. <https://doi.org/10.1111/mmi.12995>

**Published in:**  
Molecular Microbiology

**Document Version:**  
Peer reviewed version

**Queen's University Belfast - Research Portal:**  
[Link to publication record in Queen's University Belfast Research Portal](#)

### **Publisher rights**

Copyright 2015 Wiley

This is the peer reviewed version of the following article: Wong, A., Lange, D., Houle, S., Arbatsky, N. P., Valvano, M. A., Knirel, Y. A., Dozois, C. M. and Creuzenet, C. (2015), Role of capsular modified heptose in the virulence of *Campylobacter jejuni*. *Molecular Microbiology*, 96: 1136–1158, which has been published in final form at <http://onlinelibrary.wiley.com/doi/10.1111/mmi.12995/supinfo>.

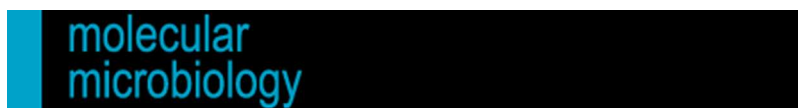
This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for self-archiving

### **General rights**

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### **Take down policy**

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact [openaccess@qub.ac.uk](mailto:openaccess@qub.ac.uk).



**Role of capsular modified heptose in the virulence of  
*Campylobacter jejuni***

Journal:	<i>Molecular Microbiology</i>
Manuscript ID:	MMI-2014-14618.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Wong, Anthony; Western University, Microbiology and Immunology Lange, Dirk; Western University, Microbiology and Immunology Houle, Sébastien; INRS-Institut Armand Frappie, Microbiology and Biotechnology Arbatsky, Nikolay; Russian Academy of Sciences, N.D. Zelinsky Institute of Organic Chemistry Valvano, Miguel; Queen's University Belfast, Centrew for Infection and Immunity Knirel, Yuriy; Russian Academy of Sciences, N.D. Zelinsky Institute of Organic Chemistry Dozois, Charles; INRS-Institut Armand Frappier, Microbiology and Biotechnology Creuzenet, Carole; University of Western Ontario, Microbiology and Immunology
Key Words:	Campylobacter, Capsule, Heptose, Virulence, Macrophages

1           **Role of capsular modified heptose in the virulence of *Campylobacter jejuni***

2  
3       Anthony Wong <sup>‡1</sup>, Dirk Lange <sup>‡1,2</sup>, Sebastien Houle <sup>§</sup>, Nikolay P. Arbatsky <sup>+</sup>, Miguel A.  
4       Valvano <sup>‡¶</sup>, Yuriy A. Knirel <sup>+</sup>, Charles M. Dozois <sup>§</sup> and Carole Creuzenet <sup>‡\*</sup>

5  
6       From the <sup>‡</sup>Department of Microbiology and Immunology, Western University, London,  
7       Ontario, Canada, <sup>§</sup>INRS-Institut Armand Frappier, Laval, Québec, Canada, <sup>¶</sup>Centre for  
8       Infection and Immunity, Queen's University of Belfast, Belfast, United Kingdom, and  
9       <sup>+</sup>N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow,  
10       Russia.

11  
12       \* **Corresponding author.** Dept. of Microbiology and Immunology, Western University,  
13       DSB 3031, London, Ontario, N6A 5C1, Canada. Tel.: 1-519-661-3204; Fax: 1-519-661-  
14       3499; E-mail: [ccreuzenet@uwo.ca](mailto:ccreuzenet@uwo.ca)

15  
16       <sup>1</sup>: **equal contribution**

17       <sup>2</sup>: **Current address:** Dr D. Lange: Jack Bell Research Centre, 2660 Oak Street,  
18       Vancouver, British Columbia, V6H3Z6, Canada.

19  
20       **Running title:** Role of modified heptose in virulence of *C. jejuni*.

21  
22       **Key words:** Campylobacter, capsule, heptose, virulence, chicken, macrophages.

23

## SUMMARY

The *Campylobacter jejuni* capsular polysaccharide is important for virulence and often contains a modified heptose. In strain ATCC 700819 (a.k.a. NCTC 11168), the modified heptose branches off from the capsular backbone and is directly exposed to the environment. We reported previously that the enzymes encoded by *wcaG*, *mlghB*, and *mlghC* are involved in heptose modification. Here, we show that inactivation of any of these genes leads to production of capsule lacking modified heptose and alters the transcription of other capsule modification genes differentially. Inactivation of *mlghB* or *mlghC*, but not of *wcaG*, decreased susceptibility to bile salts and abrogated invasion of intestinal cells. All mutants showed increased sensitivity to serum killing, especially *wcaG::cat*, and had defects in colonization and persistence in chicken intestine, but did not show significant differences in adhesion, phagocytosis, and intracellular survival in murine macrophages. Together, our findings suggest that the capsular heptose modification pathway contributes to bacterial resistance against gastrointestinal host defenses and supports bacterial persistence via its role in serum resistance and invasion of intestinal cells. Our data further suggest a dynamic regulation of expression of this pathway in the gastrointestinal tract.

## INTRODUCTION

*Campylobacter jejuni* infections are the leading cause of enteritis worldwide (Rautelin and Hanninen, 2000; Wassenaar and Blaser, 1999). Infected patients can also develop neurological complications such as Guillain-Barre and Miller-Fischer syndromes (Godschalk *et al.*, 2004; Godschalk *et al.*, 2007). Numerous factors contribute to pathogenicity of *C. jejuni* and successful host colonization, including production of toxins and glycoproteins (Guerry *et al.*, 2006; Kakuda and DiRita, 2006; Karlyshev *et al.*, 2004; Szymanski *et al.*, 2002; Vijayakumar *et al.*, 2006) and resistance to bile salts (Lin *et al.*, 2003; Lin *et al.*, 2005). Flagella-mediated motility also contributes to the virulence of *C. jejuni* (Biswas *et al.*, 2007), as it facilitates colonization of the mucus layer covering the intestinal epithelium (Beery *et al.*, 1988; Lee *et al.*, 1986) and subsequent invasion of epithelial cells (Jin *et al.*, 2001; Konkel *et al.*, 2005; Pei and Blaser, 1993; Vijayakumar *et al.*, 2006). Survival of *C. jejuni* within macrophages for several days is also a key virulence factor (Day *et al.*, 2000; Kiehlbauch *et al.*, 1985), and epithelial and macrophage cell damage resulting from invasion may be critical for the inflammatory response elicited by *C. jejuni* infection (Biswas *et al.*, 2000; Fauchere *et al.*, 1986; Manninen *et al.*, 1982; Newell and Pearson, 1984; Newell *et al.*, 1985; Szymanski *et al.*, 1995). Further, the surface expression of lipooligosaccharide (LOS), which in many *C. jejuni* strains mimics human gangliosides, has been associated with autoimmune reactions implicated in the Guillain-Barre and Miller-Fischer syndromes (Godschalk *et al.*, 2007; Mishu and Blaser, 1993; Salloway *et al.*, 1996; Yuki *et al.*, 1994).

*C. jejuni* also produces a surface capsular polysaccharide (CPS) (Karlyshev *et al.*, 2000; Karlyshev *et al.*, 2001) initially identified as a heat-stable antigen and thought to be

a lipopolysaccharide (Preston and Penner, 1989). Mutational studies have demonstrated that CPS is a virulence factor for *C. jejuni*. For example, a non-capsular *kpsE* mutant of strain ATCC 81116 shows reduced adherence and invasion of intestinal epithelial cells (Bachtiar *et al.*, 2007), and a non-capsular *kpsM* mutant of strain ATCC 81-176 has reduced invasion of intestinal epithelial cells, is attenuated in a ferret model (Bacon *et al.*, 2001), and has decreased ability to colonize the gut in chickens (Jones *et al.*, 2004). Non-capsular mutants of invasive clinical isolates 84-25 and 84-19 are also more readily killed by serum complement than wild type (Keo *et al.*, 2011).

The CPS from strain ATCC 700819 (a.k.a. NCTC 11168) used in this study consists of a phospholipid anchor (Corcoran *et al.*, 2006) and repeating units containing a backbone of  $\beta$ -D-ribofuranose ( $\beta$ -D-Ribf), 2-acetamido-2-deoxy- $\beta$ -D-galactofuranose ( $\beta$ -D-GalfNAc), and  $\alpha$ -D-glucopyranuronic acid ( $\alpha$ -D-GlcpA) amidated with 2-amino-2-deoxyglycerol (GroN) or ethanolamine (EtN) (Fig. 1A). The repeating units also comprise a side branch consisting of a modified heptose: 6-O-methyl-D-glycero- $\alpha$ -L-gluco-heptopyranose (6-O-Me-D- $\alpha$ -L-glucoHepp) (McNally *et al.*, 2007; St Michael *et al.*, 2002; Szymanski *et al.*, 2003) or 3,6-O-Me<sub>2</sub>-D- $\alpha$ -L-glucoHepp (McNally *et al.*, 2007). Addition of O-methyl phosphoramidate (MeOPN) moieties onto heptose and GalfNAc residues has also been reported (McNally *et al.*, 2007; Szymanski *et al.*, 2003). The role of MeOPN modification of CPS in virulence and host colonization is controversial. A contribution to insecticidal activity was demonstrated in one study (Champion *et al.*, 2010), but another study showed neither insecticidal activity nor any effect on chicken colonization (van Alphen *et al.*, 2014). The MeOPN modification may increase invasion to epithelial cells and resistance to serum killing (van Alphen *et al.*, 2014) or modulate

host immune responses (Maue *et al.*, 2013). The contribution to virulence of the uniquely modified heptoses remains unknown. Due to their branch location in the CPS of strain ATCC 700819, the modified heptose residues protrude into the extracellular environment, becoming the outermost exposed carbohydrates of the CPS.

Various heptose derivatives are found in the CPS of *C. jejuni* (Aspinall *et al.*, 1992; Aspinall *et al.*, 1995a; Chen *et al.*, 2008; Kilcoyne *et al.*, 2006), *C. coli* (Aspinall *et al.*, 1993) and *Burkholderia pseudomallei* (Perry *et al.*, 1995; Reckseidler *et al.*, 2001), in the exopolysaccharide of *C. lari* (Aspinall *et al.*, 1995b), and in the lipopolysaccharide of *Yersinia pseudotuberculosis* (Samuelsson *et al.*, 1974). We previously demonstrated that the modified heptoses play a role in *Y. pseudotuberculosis* virulence (Ho *et al.*, 2008; Kondakova *et al.*, 2008). Variations in the heptose structure (ring configuration and modifications) among different isolates and species suggest specific adaptations of the heptose residue to its function within the polysaccharide. We therefore hypothesized that the modified heptoses and their unusual ring configurations are important to fine tune host-bacteria interactions.

Genomic sequence data of strain ATCC 700819 suggest that the *C. jejuni* CPS is related to group II and group III CPS of other Gram-negative bacteria (Karlyshev *et al.*, 2000). CPS gene clusters of *E. coli* strains that produce type II and III CPS show a conserved organization of the genes into three regions (Boulnois *et al.*, 1987; Boulnois and Roberts, 1990). Regions 1 and 3 include genes involved in CPS export, such as the gene for the CPS ABC-transporter component KpsM for transport across the inner membrane (Cuthbertson *et al.*, 2010; Whitfield, 2006). The central region 2 comprises genes for the synthesis of serotype-specific sugars and their assembly into repeating units

(Roberts *et al.*, 1988a; Roberts *et al.*, 1988b). These features apply to *C. jejuni* strain ATCC 700819 (Fig. 1B). In the CPS cluster of this strain and also in *B. pseudomallei*, region 2 contains genes devoted to the synthesis of GDP-D-*glycero- $\alpha$ -D-manno*-heptose (Kneidinger *et al.*, 2001; Parkhill *et al.*, 2000; Reckseidler *et al.*, 2001), suggesting that this compound is likely the precursor for the synthesis of the modified heptose present in their CPS, as demonstrated previously for the 6-deoxy-D-*manno*-heptose in *Y. pseudotuberculosis* (Butty *et al.*, 2009).

We recently elucidated the biochemical synthesis pathways of the 6-deoxy-D-*altro*-heptose present in *C. jejuni* 81-176, which involved the sequential activity of the C4, C6 dehydratase DdahA (a.k.a. WcbK), a C3 epimerase DdahB (a.k.a. Cjj1430) and a C4 reductase DdahC (a.k.a. Cjj1427) on GDP-D-*glycero*-D-*manno*-heptose (McCallum *et al.*, 2011; McCallum *et al.*, 2012). This pathway is under post-translational regulation by the activity of WcaG, a C4 reductase encoded by the CPS cluster itself that reduces the DdahA product, thereby diverting it off the *altro*-heptose synthesis pathway. We also showed additional regulation of the biosynthesis pathway via interactions between WcaG and DdahC. Using this knowledge, we demonstrated the C3, C5 epimerase activity of MlghB (encoded by *mlghB*, a.k.a. *cj1430c*) and the C4 reductase activity of MlghC (encoded by *mlghC*, a.k.a. *cj1428c*) of *C. jejuni* ATCC 700819 (Fig 1B) (McCallum *et al.*, 2013). This was achieved by using a hybrid pathway that combined MlghB and MlghC from *C. jejuni* ATCC 700819 with the DdahA dehydratase from *C. jejuni* 81-176 (Fig 2) since the putative oxidase MlghA necessary to initiate the pathway in *C. jejuni* ATCC 700819 is unknown. We also showed a C4 reductase activity on the DdahA product for the WcaG enzyme encoded by *wcaG* (a.k.a. *cj1427c*) in strain ATCC 700819



(McCallum *et al.*, 2013). The proposed 6-O-methyl-D-glycero- $\alpha$ -L-gluco-heptose (called thereafter 6OMe-Hep) synthesis pathway is depicted in Fig. 2. Our biochemical work suggests that WcaG, MlghB, and MglhC are required for CPS heptose modification. This agrees with data showing that homologues of *wcaG*, *mlghB*, and *mlghC* are only found in CPS biosynthesis loci of strains whose CPS contains modified heptoses (Karlyshev *et al.*, 2005; St Michael *et al.*, 2002), and with the observation that inactivation of *mlghC* results in loss of the modified heptose (St Michael *et al.*, 2002).

In this study, we investigated the role of *wcaG*, *mlghB*, and *mlghC* genes from strain ATCC 700819 in CPS synthesis and function. Using a panel of defined mutants, we determined the contribution of each gene to CPS composition and to phenotypes associated with pathogenesis including protection against bile salts, detergents and serum, and bacterial motility. We also tested the contribution of these genes to *C. jejuni*'s adhesion and invasion of intestinal epithelial cells, survival within macrophages, and colonization of the chicken intestine. Our results demonstrate that *wcaG*, *mlghB*, and *mlghC* are required for CPS heptose modification and indicate that the modified heptoses contribute to the pathogenicity of *C. jejuni* ATCC 700819.

## RESULTS

### *Genetic characterization of the capsular heptose biosynthesis mutants*

Insertional mutants in *wcaG*, *mlghB*, *mlghC*, and *kpsM* genes were constructed as described under *Experimental Procedures*. PCR and Southern blot analyses indicated that each mutant contained the disrupted targeted gene without compromising the surrounding

genes (data not shown). One of the *wcaG::cat* mutants, named *wcaG::catΔ*, also had a 6.1-kb deletion removing genes *cjl421c* to *cjl426c*. This mutant was included in our studies for the following reasons. First, the deletion removes *cjl425c-cjl423c*, which are involved in the synthesis of the heptose precursor (GDP-*glycero-manno*-heptose, Fig.1B) (Karlyshev *et al.*, 2005; Kneidinger *et al.*, 2001; Parkhill *et al.*, 2000; Valvano *et al.*, 2002), suggesting that *wcaG::catΔ* should lack heptose in its CPS. Second, the deletion also eliminates the putative heptose methyltransferase *cjl426c* (Fig.1B) (Sternberg *et al.*, 2013), thereby ensuring lack of methylation. Third, *cjl422c* and *cjl421c*, which are also deleted, encode enzymes for the transfer of MeOPN to D- $\alpha$ -L-*glucoHepp* and  $\beta$ -D-GalNAc, respectively (Fig.1B) (Karlyshev *et al.*, 2005; McNally *et al.*, 2007). While MeOPN synthesis genes (*cjl415c-cjl418c*) are intact, the attachment of MeOPN to the CPS should not occur in this mutant. Therefore, *wcaG::catΔ* should produce a CPS backbone devoid of modifications (no heptose, no methylation and no MeOPN), thereby serving as a valuable control in our study. The *kpsM* mutant provided a non-capsular, negative control since KpsM is a critical component of the ABC transporter for CPS export across the cytoplasmic membrane (Silver *et al.*, 2001). Complemented strains were constructed by re-introducing the original genes in the chromosome between the 16S and 23S rRNA genes and using the constitutive OmpE promoter as described previously (Karlyshev and Wren, 2005).

For all phenotypic analyses, the strains were revived under appropriate antibiotic selection, but the final sub-culture prior to phenotypic analysis was performed in the absence of antibiotic selection so that all strains were grown under the exact same conditions. Bacteria were harvested and suspensions normalized to the same OD<sub>600nm</sub> to

alleviate potential variation arising from different growth rates. CFU measurements performed on bacterial suspensions used as inoculum for phenotypic assays indicated similar viability of all strains used as measured after 24 h of growth (data not shown).

### *Mutants in the heptose modification pathway produce high molecular weight CPS*

The effect of inactivation of heptose modification genes on CPS synthesis was investigated by SDS-PAGE and silver staining of CPS obtained by hot water/phenol extraction (Westphal and Jann, 1965) and ultracentrifugation. The wild type had high and low molecular weight bands (Fig. 3A) corresponding to CPS and co-extracted LOS, respectively (Karlyshev and Wren, 2001). As expected, the *kpsM* mutant lacked high molecular weight bands while production of LOS was not affected (Fig. 3A). In contrast, all heptose modification mutants formed CPS but *mlghB::cat* apparently produced less CPS than the others (Fig. 3A). Western blot analysis (with anti HS:2 Penner serotyping antibodies) of CPS samples obtained by SDS solubilization of total cells and proteinase K digestion of proteins (Hitchcock and Brown, 1983) also showed that all mutants tested produced CPS (Fig. 3B) and the amount produced by *mlghB::cat* was on par with all other strains. The differences in relative amounts seen for *mlghB::cat* by silver staining and Western blotting could relate to slight variations in CPS composition that could affect silver staining (Szymanski et al., 2003) and CPS solubility and recovery in the hot water/phenol extraction method, but compositional data below exclude this possibility. The differences probably rather relate to additional modifications of surface properties in this mutant, which may in turn affect extractability of the CPS by the hot water/phenol

method. Together, these results indicate that the absence of heptose or modified heptose does not prevent production of high molecular weight CPS.

Slight upwards shifts of CPS bands of the mutants were observed depending on loading amounts and gel composition. Because wild type *C. jejuni* CPS is anchored to the outer membrane via a phospholipid (Corcoran *et al.*, 2006), the band shifts could reflect CPS anchoring to a different lipid carrier, such as lipid A, as seen in *E. coli* K<sub>LPS</sub> (Jann *et al.*, 1992). To test this possibility, CPS samples were treated with phospholipase prior to SDS-PAGE analysis (Karlyshev *et al.*, 2000). This led to disappearance of CPS bands in all strains tested (data not shown), demonstrating that CPS was phospholipid-bound. Also, in contrast to LOS bands, wild type and mutant CPS bands did not react with anti-lipid A antibody (data not shown), confirming that CPS was not covalently linked to lipid A. We conclude that the band shifts represent subtle changes in CPS composition affecting not only the size of CPS units but also their hydrophobicity and ability to bind SDS.

*The CPS of heptose modification pathway mutants does not contain methylated heptose*

The composition of hot water/phenol extracted CPS was determined using a sugar analyzer after complete acid hydrolysis, and also using one- and two-dimensional NMR spectroscopy (Tables 1 and 2, Figs. S1 and S2). The data obtained, combined with data reported for *C. jejuni* NCTC 11168 CPS (McNally *et al.*, 2007; St Michael *et al.*, 2002; Szymanski *et al.*, 2003), enabled inferring the CPS structures of wild type and mutant strains (Fig. 4). Sugar analysis revealed the three CPS backbone constituents, Rib,

GalNAc (detected as GalN) and GlcA in all strains. The higher than expected Rib/GalN ratios (expected equimolar) are likely due to RNA contamination of the samples. GlcA was not quantitated because it was amidated with EtN or GroN and was poorly released by hydrolysis but NMR spectroscopy analyses confirmed its presence by the characteristic signals for H-1 and C-1 in all strains (Table 2). Without authentic D-*glycero*-L-*gluco*-heptose as standard, the presence or absence of heptose was inferred from signals for H-1 at  $\delta$  5.58 and C-1 at  $\delta$  98.3, which were observed in  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of wild type CPS only (Table 2). Characteristic signals were also detected for 3-O-Me and 6-O-Me groups on heptose in wild type only (Fig S2), confirming that heptose was present and 3,6-di-O-methylated in wild type CPS while it was absent in the CPS from the mutants.

NMR spectroscopy did not reveal MeOPN modification in wild type CPS, but MeOPN was readily apparent on GalNAc in *wcaG::cat* and *mlghB::cat* (Table 2) and traces of MeOPN were found in *mlghC::cat*. Consistent with the deletion of MeOPN transfer genes in *wcaG::cat* $\Delta$ , no MeOPN was detected in its CPS despite the presence of the GalNAc acceptor. Also, while the GlcA residue carried only the EtN substituent in wild type CPS, both EtN and GroN were found in the mutants (Table 1). These differences between wild type and heptose modification mutants could represent a compensatory mechanism whereby amidation of GlcA by GroN and/or MeOPN addition on GalNAc is enhanced when the production of D-*glycero*-L-*gluco*-heptose is impaired. No O-methyl groups were present in the complemented *wcaG* and *mlghC* mutants by NMR spectroscopy, suggesting that incorporation of wild type-like modified heptose was

not restored in these strains. The complemented *mlghB* strain was not tested by NMR spectroscopy for lack of sufficient CPS material.

### *Inactivating heptose modification genes causes drastic changes in transcription within the CPS gene cluster*

The compensatory mechanisms inferred from NMR spectroscopy analyses in our mutants and lack of complementation of modified heptose incorporation in the CPS could result from polarity effects or from transcriptional regulation within the CPS cluster. We used quantitative real-time PCR (qRT-PCR) to demonstrate lack of polarity on expression of genes located downstream of the chloramphenicol (*cat*) and kanamycin (*kan*) resistance cassettes used for gene inactivation. Expression of the gene immediately downstream of *kpsM* in *kpsM::kan* or downstream of *wcaG* and *mlghC* in *wcaG::cat* and *mlghC::cat*, respectively was not affected (Fig. 5), indicating that insertion of the *cat* and *kan* cassettes was non-polar. However, in *mlghB::cat*, transcription of *cj1429c* directly downstream of *mlghB* was upregulated ~187-fold. Since the *cat* cassette causes no polarity, we concluded that the upregulation of *cj1429c* transcription is related to inactivation of *mlghB* via a regulatory effect and not a mutagenesis artifact. Such transcriptional regulation implies the existence of promoters within the CPS cluster.

*C. jejuni* promoters are different from *E. coli* promoters (Wosten *et al.*, 1998) and difficult to predict. To test for their existence, we measured the transcription levels of genes involved in CPS synthesis and modification by qRT-PCR. By doing so, we could also assess whether inactivation of one step during D-manno- to L-gluco- heptose

conversion had any feedback regulatory effects on other steps or on other CPS modifications (e.g. MeOPN addition, amidation of GlcA, heptose methylation). Measurements were relative to housekeeping gene *cj1537c* (acetyl-CoA synthase) and accounted for primer efficiencies.

Expression levels varied considerably among the genes tested, with some too low to be quantitated accurately (e.g. *kpsM*), others on par with the housekeeping control, and others ~15-fold greater than control (Fig. 5A). The various transcription patterns observed indicate that numerous promoters exist within the CPS cluster. To further assess potential feedback regulatory effects of inactivation of one gene on surrounding genes, we analyzed the data as expression ratios between each mutant and the wild type (Pfaffl, 2001) (Fig. 5B). This comparison indicated that inhibition of CPS transport by *kpsM* inactivation only had minor effects on heptose modification genes and no effects on the remaining CPS cluster genes tested, and that inactivation of biosynthesis/modification genes had little or no impact on transport functions. However, inactivation of *mlghB* or *mlghC* (responsible for heptose ring configuration switch from D-manno to L-gluco) led to upregulation of other genes involved in heptose synthesis and modification, including the O-methyl-transferase gene *cj1426c* (2.8- to 9.9-fold). Inactivation of *mlghB* or *mlghC* also strongly upregulated the transcription of *cj1429c* (186.7- and 18.1-fold, respectively), a gene of unknown function. Inactivation of *wcaG* produced different effects, with modest upregulation of heptose modification genes (2.9- to 4.5-fold), no effect on heptose synthesis and 60% reduction of transcription of *cj1429c*. These differences could be due to the previously established regulatory role of WcaG on heptose modification by substrate scavenging and interaction with MlghC (McCallum *et*

al., 2011; McCallum *et al.*, 2012; McCallum *et al.*, 2013). Together, the transcriptional data suggest that inactivation of *wcaG*, *mlghB*, or *mlghC* or the absence of their products exert regulatory effects on other CPS modification genes, which may influence the functional properties of CPS.

We next assessed whether these regulatory effects were abrogated in our complemented strains, focusing on the *mlghC* and *mlghB* mutants that showed the strongest regulatory effects above. Reintroduction of *mlghC* into the *mlghC* mutant led to 60 $\pm$ 1.5 fold up-regulation of *mlghC* compared to wild type, mirrored by 67 $\pm$ 3.8 fold up-regulation of *mlghB* upon its reintroduction into the *mlghB* mutant. While such high transcription levels had no significant effects on transcription of other CPS genes such as *wcaG* and *mlghB* in the complemented *mlghC* strain (with 1.9 $\pm$ 0.3 and 0.4 $\pm$ 0.1 fold compared to wild type, respectively), *wcaG* and *mlghC* were upregulated 5.4 $\pm$ 0.3 and 8.5 $\pm$ 0.4 fold compared with wild type, respectively, in the complemented *mlghB* strain, which may affect CPS composition and function. Overall, this analysis indicates that the strong OmpE promoter and internal promoters in the CPS cluster result in significant transcriptional differences in complemented strains, which together with enzymatic regulatory loops (McCallum *et al.*, 2013) may explain the lack of functional complementation of the mutants observed by NMR. Others have also reported previously similar difficulties with complementation upon perturbation of regulatory networks or improper stoichiometry of enzymes involved in polysaccharide synthesis (van Sorge *et al.*, 2014). Therefore, in view of these difficulties and since qRT-PCR data demonstrated lack of polarity of the mutations, we did not pursue the characterization of the “complemented” strains.



319  
320 *Disruption of heptose modification pathway affects growth rate and decreases motility*  
321 *but does not affect autoagglutination*  
322  
323 When measuring growth rates of wild type and mutants under microaerobic conditions,  
324 we observed that the heptose modification mutants grew consistently faster than wild  
325 type. The *wcaG::catΔ* mutant, which not only lacks heptose in its CPS but also lacks  
326 MeOPN on GalNAc (Fig. 6A), reached the highest final cell density. This suggests that  
327 implementation of the heptose modification pathway (configuration switch, and  
328 methylation) and of the MeOPN addition pathway imposes an energetic cost. The  
329 *kpsM::kan* mutant exhibited a longer lag phase before entering exponential phase,  
330 suggesting that the accumulation of CPS units or CPS precursors inferred from the qRT-  
331 PCR analysis above may be slightly toxic to the bacterium, as observed in comparable  
332 mutants of other species (Kroll *et al.*, 1988; Pavelka *et al.*, 1994).  
333 We also investigated if alteration of CPS amount or composition in the mutants  
334 could affect autoagglutination, which was measured as the decrease of turbidity over time  
335 in the upper level of static bacterial suspensions. Autoagglutination occurred in all strains  
336 and there was no significant difference between wild type and the heptose modification  
337 mutants (Fig. 6B). In contrast, *kpsM::kan* showed increased autoagglutination (Fig. 6B)  
338 and microscopy revealed that this mutant formed clumps of cells (Fig. 6C). Motility was  
339 assessed in soft agar to determine if impaired motility contributed to the strong  
340 autoagglutination of *kpsM::kan*. This mutant was nonmotile while the heptose  
341 modification mutants were motile, albeit at a statistically significantly reduced rate

342 compared to wild type (Table 3). Electron microscopy revealed that the majority of  
343 *kpsM::kan* cells produced flagella similar to the wild type strain (Fig. 6D), suggesting that  
344 loss of motility was due to a defect in flagellar function. Overall, the total lack of motility  
345 of *kpsM::kan* may contribute to its autoagglutination.

#### 347 *Disruptions of heptose modification pathway alters CPS barrier function*

348  
349 CPS contributes to resistance to bile salts in many bacteria (Begley *et al.*, 2002; Hsieh *et*  
350 *al.*, 2003; Pace *et al.*, 1997). We tested whether CPS and its modified heptose contribute  
351 to resistance to bile salts for *C. jejuni*. Wild type and mutant strains were exposed to  
352 increasing concentrations of a mixture of cholic and deoxycholic acids, two abundant bile  
353 salts in humans. Low concentrations (0-0.25 g/l) of bile salts had little effect on all strains  
354 (Fig. 7A). However, higher concentrations (0.5-2.0 g/l) resulted in 80-90% killing in the  
355 wild type strain. Interestingly, *kpsM::kan* was as susceptible to bile salt killing as wild  
356 type, indicating that CPS as a whole and in its wild type structure and composition does  
357 not contribute to resistance. Likewise, *wcaG::catΔ* and *wcaG::cat* had wild type like bile  
358 salts susceptibility while *mlghC::cat* and *mlghB::cat* were more resistant ( $p < 0.001$ ) than  
359 wild type. These differences in bile salts resistance may reflect subtle differences in the  
360 CPS, which may not be apparent in NMR analyses of CPS extracted from the mutants  
361 since the mutants had similar NMR profiles, except for a lower MeOPN content in  
362 *mlghC::cat* and lack of MeOPN in *wcaG::catΔ*. The observed differences for *mlghB::cat*  
363 and *mlghC::cat* were specific to bile salts, as no differences in resistance to SDS were  
364 observed for these mutants or with any of the others.

CPS protects bacteria against killing by serum, including in *C. jejuni* 81-176 (Maue *et al.*, 2013). We investigated whether alterations of CPS composition in our mutants would affect resistance to serum killing. The wild type exhibited 40% survival after 90 min in 30% serum, while non-capsular *kpsM::kan* did not survive in 5% serum (Fig. 8A), indicating that wild type CPS protects *C. jejuni* against serum killing. The *wcaG::cat* and *wcaG::catΔ* mutants were resistant to up to 5% serum but highly susceptible to concentrations above 20%, while *mlghB::cat* and *mlghC::cat* displayed intermediate susceptibility, resisting killing up to ~15% of serum, but being killed by more than 30% serum (Fig. 8A). Time-course experiments were performed using 20% serum, a concentration where maximal differences were observed between strains (Fig. 8B). The *kpsM::kan* and *wcaG::catΔ* mutants were killed very rapidly while *wcaG::cat* resisted slightly longer (Fig. 8B). Together, these results demonstrate that heptose in its 3,6-O-Me<sub>2</sub>-L-*gluco* form, as present in wild type, is essential for CPS-mediated protection against serum killing. In contrast, *mlghC::cat* showed wild type killing kinetics for the first 45 min, with a slightly lower survival afterwards (Fig. 8B). Since the only difference between the CPS of *mlghC::cat* and *wcaG::cat* is the lower amount of MeOPN in *mlghC::cat*, we conclude that MeOPN is actually deleterious for resistance to serum within the context of heptose-less mutants. This agrees with the observation that wild type does not produce MeOPN under our growth conditions. Unexpectedly, *mlghB::cat* resisted killing better than all strains including wild type for about 45 min, but plummeted beyond that time to reach wild type levels at 90 min. Because we could not distinguish the CPS of *mlghB::cat* from that of *wcaG::cat* by NMR spectroscopy or Western blotting,

their different serum resistance may reflect additional differences in surface properties in the *mlghB::cat* mutant.

*Disruption of heptose modification pathway does not affect interactions with RAW 264.7 macrophages*

Several studies have suggested that *C. jejuni* can survive within murine macrophages for several days (Day *et al.*, 2000; Kiehlbauch *et al.*, 1985; Mixter *et al.*, 2003). To test whether the CPS and its modified heptose protect *C. jejuni* against intracellular killing, the CPS mutants were assayed for survival in RAW 264.7 macrophages. Intracellular survival decreased drastically within a few hours and there was no statistically significant difference between wild type and any of the mutants whether the data were analyzed as percent of original inoculum (Fig. S3A) or percent of initial bacterial load (Fig. S3B). Likewise, no significant differences were observed between heptose modification mutants and wild type regarding adhesion and phagocytosis rates (Fig. S3C and S3D). These data indicate that the modified heptose does not protect against adhesion, uptake or clearance by macrophages. A slight anti-phagocytic role could nevertheless be attributed to wild type CPS as *kpsM::kan* was engulfed at higher levels than wild type when interactions were allowed to proceed for at least 2 h (Fig S3D).

*The heptose modification pathway is important for invasion of intestinal epithelial cells*

Adhesion to intestinal epithelial cells and their invasion are important for *C. jejuni* pathogenicity (Black *et al.*, 1988; De Melo *et al.*, 1989; Fauchere *et al.*, 1986; Konkel *et al.*, 1992) and CPS is thought to be essential for these processes (Bachtiar *et al.*, 2007; Bacon *et al.*, 2001). We therefore assessed our mutants for adhesion and invasion with Caco-2 intestinal epithelial cells. The heptose modification mutants adhered at wild type levels, indicating no role of heptose modification in adhesion (Fig. 9A). Differences among heptose modification mutants were observed for invasion, with abrogated invasion for *mlghB::cat* and *mlghC::cat*, wild type invasion for *wcaG::cat*, and increased invasion for *wcaG::catΔ*. Finally, *kpsM::kan* was more adhesive and invasive than wild type. Phase contrast microscopy showed that it agglutinated on the surface of Caco-2 cells, which could enhance adhesion, while other mutants did not (data not shown).

*The heptose modification pathway is important for colonization and persistence in chicken intestine*

*C. jejuni* wild type and mutants were tested for their ability to colonize the intestine of two day old chicks. All mutants were significantly impaired for colonization and/or persistence in the chicken intestinal tract compared to wild type, but differences were also observed between the mutants (Fig. 10). Specifically, *wcaG::catΔ* and *mlghC::cat* demonstrated a median 5.4 log ( $p < 0.0001$ ) and 6.3 log ( $p < 0.0001$ ) reduction in colonization compared to wild type, respectively. In addition, while infection of all inoculated chicks was observed with wild type, *wcaG::catΔ* and *mlghC::cat* were only isolated from 6 of 11 (54.5%) and 4 of the 12 (25%) inoculated chicks, respectively. In

contrast, bacteria were isolated from all chicks infected with *mlghB::cat* and *kpsM::kan* and in 12 out of 14 chicks inoculated with *wcaG::cat*. All these mutants also persisted significantly less than wild type and demonstrated medians of 1.9 log (p=0.0001), 1.1 log (p=0.0231) and 1.8 log (p=0.0095) reduction in colonization when compared to wild type, for *mlghB::cat*, *kpsM::kan* and *wcaG::cat* respectively. The data indicate that in *C. jejuni* ATCC 700819, CPS is important but not essential for colonization of the chick intestine. We speculate that underlying adhesins compensate for the lack of CPS. However, when CPS is present, its wild type composition featuring the modified heptose appears optimal for colonization of the chicken intestinal tract.

## DISCUSSION

To assess the role of the role of heptose and its modification in CPS function, we performed a detailed analysis of *C. jejuni* mutants of the *wcaG*, *mlghB*, and *mlghC* genes, which affect the switch of CPS heptose from a D-manno to a L-gluco form (McCallum *et al.*, 2013). Our results showed that these genes play unexpected roles in transcriptional regulation within the CPS cluster. The *C. jejuni* CPS cluster is similar to group II and III CPS clusters of *E. coli*, whereby conserved CPS transport regions 1 and 3 flank the sugar synthesis region 2 (Karlyshev *et al.*, 1999; Whitfield and Roberts, 1999). Region 3 in the *E. coli* K5 CPS cluster has a  $\sigma 70$  promoter (upstream of *kpsM* included in this region) and RfaH-dependent read-through transcription from this promoter into region 2 is essential for CPS production (Rowe *et al.*, 2000; Stevens *et al.*, 1997). If this also applied to *C. jejuni*, genes from regions 2 and 3 would be transcribed at similar levels or with

decreasing levels as their distance from the region 3 promoter increases, without differential regulation of gene expression within region 2. However, we observed differential transcription of CPS modification genes in the wild type strain, which is likely important to fine tune the CPS composition as *C. jejuni* passes from one host to another or is exposed to the environment, as the modified heptose, MeOPN and methylation may each enhance resistance to different adverse conditions. The qRT-PCR data imply the existence of internal promoters within regions 2 and 3, which agrees with a recent RNA-seq study (Dugar *et al.*, 2013). Transcriptional start sites were identified by RNA-seq upstream of *cj1445c*, *cj1444c*, and *cj1425c* to *cj1429c*, which was consistent with our qRT-PCR findings. This RNA-seq study identified a start site for *cj1447c* within *kpsM* in two strains but not in the strain NCTC 11168 used in our study. We show that transcription of *kpsM* can vary without affecting that of *cj1447c*, indicating that the start site for *cj1447c* is also present in strain NCTC 11168.

#### *Implications of heptose modification pathway disruption for CPS composition*

While it had already been shown that a *mlghC::cat* mutant produces heptoseless CPS (St Michael *et al.*, 2002), as we also observed in our study, the CPS composition in *wcaG::catΔ*, *wcaG::cat* and *mlghB::cat* mutants had to be determined. We found no heptose in *wcaG::catΔ* by NMR spectroscopy analysis, as expected from deletion of its heptose biosynthesis genes. Like for *mlghC::cat*, we also found no modified heptose in *wcaG::cat* and *mlghB::cat*. The lack of even an unmodified heptose in these mutants

despite their ability to synthesize GDP-D-*glycero*-D-*manno*-heptose is consistent with the generally high specificity of glycosyltransferases involved in CPS assembly.

SDS-PAGE analysis indicated that except for *kpsM::kan*, CPS production was not abrogated in the mutants, and lack of heptose did not prevent CPS polymerization.

Further, all mutants produced comparable amounts of CPS, although lower hot water/phenol extractability of the CPS produced by *mlghB::cat* was observed. This difference in CPS extractability along with its enhanced serum resistance despite equal CPS composition and production suggest additional differences in surface properties in this mutant. It is possible that such additional differences could be mediated by the striking ~187 fold upregulation of *cj1429c* that is observed in this mutant only.

The upregulation of the putative heptose methyl-transferase gene in all mutants shown by qRT-PCR did not result in incorporation of methyl heptose in the CPS, as shown by NMR spectroscopy. This could indicate that either methylation occurs after the heptose configuration switch or that methylated *manno*-heptose is not recognized as a substrate by glycosyltransferases for incorporation into CPS. NMR spectroscopy analysis also showed that the MeOPN modification was strongly upregulated in heptose biosynthesis mutants, which may affect the biological function of their CPS.

#### *Role of modified heptose on barrier function of CPS*

*C. jejuni* can resist physiological levels of bile salts (0.1-21 mM) (Lin *et al.*, 2003; Lin *et al.*, 2005) and has been isolated from the gall bladder and bile (Gerritsen van der Hoop and Veringa, 1993; Udayakumar and Sanaullah, 2009). In *Listeria monocytogenes* and



500 *Vibrio parahaemolyticus*, upregulation of CPS genes upon exposure to bile salts suggests  
501 a role of CPS in bile resistance (Begley *et al.*, 2002; Pace *et al.*, 1997). Therefore, we  
502 determined whether CPS as a whole, or its modified heptose, contributes to resistance of  
503 *C. jejuni* to bile salts. The concentrations tested (0.25 to 2 g/l) amount to ~ 0.6 to 4.8 mM  
504 and fall within the physiological ranges previously reported (Campbell *et al.*, 2004; Lin *et*  
505 *al.*, 2003; Perez de la Cruz Moreno *et al.*, 2006). The wild type-like susceptibility of  
506 *kpsM::kan* suggests that wild type CPS does not provide resistance against bile salts, and  
507 the enhanced resistance of the *mlghB* and *mlghC* mutants showed that heptose and its  
508 modification as present in wild type CPS actually rendered the strain more susceptible to  
509 bile salts. Therefore, the cumulative effects of lack of heptose and methylation, and  
510 variations in MeOPN, GroN and EtN contents observed in the mutants, could alter the  
511 bacterial surface hydrophobicity and confer slight protective function to CPS via  
512 enhanced repulsion of bile salts.

513       Serum complement is an essential innate immune defense against pathogens and  
514 the sensitivity of *C. jejuni* strain 81-176 to serum is mitigated by its CPS (Maue *et al.*,  
515 2013) as also reported for *Salmonella typhi*, *Klebsiella pneumoniae*, and *B. pseudomallei*  
516 (Cortes *et al.*, 2002; Hashimoto *et al.*, 1993; Reckseidler-Zenteno *et al.*, 2005). Our  
517 *kpsM::kan* mutant confirmed an essential role of CPS in protection against serum in our  
518 strain, and the heptose modification mutants showed that the modified heptose in its wild  
519 type form is essential for protection. Killing of *C. jejuni* by serum involves activation of  
520 the classical complement pathway (van Alphen *et al.*, 2014) but we do not know if *C.*  
521 *jejuni* CPS and modified heptose interfere with deposition of critical C3 complement  
522 component as observed in other pathogens (Vogel *et al.*, 1997; Woodman *et al.*, 2012).

As mentioned above, the slower kinetics of killing of *mlghB::cat* at low serum concentration may be due to additional modifications in surface properties since no obvious differences were seen in its CPS. Also, we showed a slightly deleterious effect of MeOPN, which increased serum susceptibility in the absence of heptose. Thus the role of MeOPN in serum resistance appears to depend on other CPS components, which may explain prior conflicting results (Maue *et al.*, 2013; van Alphen *et al.*, 2014). Finally, the protective function of CPS against serum killing may depend on other surface components such as LOS since a LOS mutant lacking sialic acid is serum sensitive despite the presence of CPS in strain 81-176 (Guerry *et al.*, 2000; Guerry *et al.*, 2002). All our mutants produce LOS and the serum sensitivity of *kpsM::kan* (that produces LOS but no CPS) shows that LOS does not confer protection against serum killing in strain ATCC 700819. This is consistent with prior studies indicating no role of LOS in serum resistance (Bacon *et al.*, 2001; Keo *et al.*, 2011).

#### *Role of CPS and modified heptose on interactions with macrophages and epithelial cells*

In contrast to prior reports (Day *et al.*, 2000; Kiehlbauch *et al.*, 1985; Mixter *et al.*, 2003), we did not observe prolonged survival of *C. jejuni* in murine RAW 267.4 macrophages and wild type capsule production did not affect intracellular survival or initial adhesion but decreased phagocytosis as determined by comparing wild type to non-capsular *kpsM::kan*. The sharp increase in phagocytosis of *kpsM::kan* coincided with its agglutination and likely resulted from phagocytosis of clumps of bacteria. It could also be due to exposure of adhesins that are masked by the CPS layer in wild type.

The heptose-modification mutants showed that heptose modification and MeOPN addition (that also varies in these mutants) played no role in macrophage / bacteria interactions. Thus, the true biological role of CPS modified heptose is not resistance to clearance by murine macrophages. Different effects may be observed with human or avian macrophages, and may explain the host-dependent pathogenic versus commensal character of *C. jejuni*.

We showed that *C. jejuni* ATCC 700819 adhered to and invaded Caco-2 cells at a very low rate, consistently with prior reports (Ashgar *et al.*, 2007; Carrillo *et al.*, 2004; Everest *et al.*, 1992; Ganan *et al.*, 2010; Szymanski *et al.*, 1995; Vijayakumar *et al.*, 2006). In contrast to what was observed in strain ATCC 81-176 (Bacon *et al.*, 2001), our non-capsular *kpsM::kan* mutant showed increased adhesion and invasion of Caco-2 cells (Fig. 9). Unmasking of adhesins and autoagglutination may each contribute to this phenomenon. The presence of CPS, which limits adhesion to low levels, may prevent overt tissue damage that could lead to faster bacterial clearance.

Although heptose is essential for adhesion of pathogenic *E. coli* to HeLa cells (Benz and Schmidt, 2001), our data indicate that *C. jejuni* CPS heptose is not important for adhesion to Caco-2 cells. Variable levels of invasion were observed for the heptose modification mutants but did not correlate directly with NMR structural data concerning heptose and MeOPN contents. For example, abrogated invasion for *mlghB::cat* and *mlghC::cat* could suggest a role for modified heptose in cell invasion, but the wild type-like and enhanced invasion of heptoseless *wcaG::cat* and *wcaG::cat* $\Delta$ , respectively, does not support this. Likewise MeOPN presence or abundance could not be correlated with invasion, although MeOPN contributes to epithelial cell invasion in strain 81-176 (van

Alphen *et al.*, 2014). The variations of invasion however correlated well with the levels of transcription of *cjl429c* in the mutants, and may be due to additional surface differences mediated by *cjl429c*. The increased invasion of *wcaG::catΔ* despite its lack of heptose and MeOPN also suggests that additional components contribute to the process. The data nevertheless indicate that interferences with heptose modification affect invasion. Since invasion can promote tissue damage, transepithelial transport and protection against clearance, our data suggest that the heptose modification pathway is important for host colonization and pathogenicity.

#### *Role of CPS and modified heptose on colonization of chicken intestinal tract*

The chick intestinal colonization data with *kpsM::kan* showed that CPS of strain 700819 is not essential to establish an infection since all chicks were colonized. This agrees with data obtained with a CPS export mutant of strain ATCC 81116 (Bachtiar *et al.*, 2007). These data do not negate a role for CPS in colonization but may reflect the fact that unmasked adhesins could compensate for lack of CPS. In addition, our data with *kpsM::kan* showed that CPS was important for persistence since a 1.6 log reduction of caecum load was observed. This attenuation differs from another study which showed that a *kpsM* mutant could not persist at all in the chicken intestine (Jones *et al.*, 2004). The discrepancy could be due to the use of a hypermotile strain (NCTC 11168H) and of 2-week-old chickens that present a more mature gut microflora.

Flagella-mediated motility enhances intestinal colonization by *C. jejuni* (Biswas *et al.*, 2007; Wassenaar *et al.*, 1997) but is not essential (Biswas *et al.*, 2007; Wassenaar

*et al.*, 1993) as confirmed by colonization of all chicks tested by our non-motile *kpsM::kan*. The flagellar apparatus also serves as a secretion system for *C. jejuni* invasion antigens (Cia), which facilitate colonization and persistence (Konkel *et al.*, 1999; Konkel *et al.*, 2004). The *kpsM::kan* mutant assembled flagella, indicating that it has the apparatus required for Cia secretion and should thus be able to colonize and persist within the intestinal tract as observed. Also, the increased exposure of adhesins in the absence of CPS may compensate for the lack of motility of this mutant.

The data obtained for our heptose modification mutants show a role of modified heptose in colonization and persistence. Indeed *wcaG::catΔ* and *mlghC::cat* were impaired both in colonization (less chicks colonized than wild type) and persistence (lower load than wild type), while *wcaG::cat* and *mlghB::cat* showed mostly defects in persistence (most chicks colonized but at lower levels) (Fig. 10). We assume that the effects are due to the modified heptose independently of MeOPN since a MeOPN transferase mutant of strain 81-176 showed wild type-like chicken colonization (van Alphen *et al.*, 2014), although strain-specific differences cannot be excluded. While all heptose modification mutants were less motile than wild type, we exclude that their decreased colonization was due to decreased motility since the *kpsM::kan* data indicated that the contribution of motility to colonization was limited in this model. Also, the bacterial suspensions used for inoculation were adjusted to the same OD to alleviate interferences from different growth rates, and the highest bacterial load was actually recovered with the slowest growing strain (*kpsM::kan*). Thus factors other than growth and motility were critical for gut colonization and persistence in our heptose mutants.

Overall, our data indicate that CPS and its heptose affect the efficiency of chicken gut colonization and persistence. It is likely that *C. jejuni* modulates CPS expression and composition *in vivo* at different stages of colonization as seen in other intestinal pathogens such as, for example, the Vi-antigen of *S. typhi* (Jones *et al.*, 2004). This is plausible for *C. jejuni* as expression of CPS is phase variable (Bacon *et al.*, 2001) and co-culture of *C. jejuni* with epithelial cells reduces surface CPS expression, thereby highlighting the dynamic role of CPS in interactions with host cells (Corcionivoschi *et al.*, 2009). Our data suggest that suppressed CPS expression during infection exposes surface adhesins that lead to increased agglutination of *C. jejuni*, facilitating adhesion in clumps and increasing invasion. Resumed CPS production and fine-tuning of its composition may favor persistence at other stages of infection, affecting differentially its barrier functions and its effects on intracellular survival. Our discovery of internal promoters that likely allow transcriptional optimization of CPS composition in response to environmental stimuli is in line with this hypothesis.

Collectively, the data presented in this study indicate a role of *wcaG*, *mlghB*, and *mlghC* in determining CPS composition and biological function. The mutants demonstrate very different behaviors in terms of CPS barrier functions, interactions with different cell types and motility, which culminate in different outcomes concerning colonization and persistence in the chicken intestinal tract. Overall, fine tuning of CPS composition via modified heptose incorporation and other modifications that seem to be interdependent with heptose modification, allows reaching an optimal configuration that contributes to survival of the bacterium when confronted with various host defenses along the gastrointestinal tract (bile salts, serum and phagocytic cells) and also

contributes to persistence of the bacterial population via invasion of intestinal cells. This investigation illustrates the impact that altering CPS composition can have on CPS function and on the virulence of encapsulated bacteria and suggests that the enzymes responsible for the biosynthesis of CPS components such as the modified heptose could be suitable targets for future therapeutic research.

## EXPERIMENTAL PROCEDURES

### *Bacterial culture conditions*

All experiments were carried out with strain NCTC 11168 / ATCC 700819 (HS: 2 Penner serotype). *C. jejuni* was grown in a microaerobic atmosphere (85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% O<sub>2</sub>) at 37°C on Trypticase Soy Agar (TSA) supplemented with 5% sheep blood and 10 µg/ml vancomycin and 5 µg/ml trimethoprim as background antibiotics. Where appropriate, the medium was supplemented with 15 µg/ml chloramphenicol or 30 µg/ml kanamycin. Unless stated otherwise, *C. jejuni* was grown overnight from freezer stock on TSA containing appropriate antibiotics, followed by a further growth on TSA containing only the background antibiotics for 20-24 hours. The cells were harvested and normalized to the same OD<sub>600nm</sub> before use for phenotypic analyses. Viability of the bacterial suspensions used as inoculum for phenotypic assays was assessed by CFU measurements. For transformation, *C. jejuni* was grown on Mueller Hinton (MH) agar (BD, Canada), containing 10 µg/ml vancomycin, 5 µg/ml trimethoprim, 0.05% pyruvate, and 5% heat inactivated Fetal Bovine Serum (Sigma Aldrich). *E. coli* strain DH5α was used for

cloning and was grown in Luria Bertani medium. When necessary, 100 µg/ml ampicillin, 30 µg/ml kanamycin, or 34 µg/ml chloramphenicol were added.

#### *Preparation of knockout constructs*

The *wcaG* (a.k.a. *cj1427c*), *mlghC* (a.k.a. *cj1428c*), *mlghB* (a.k.a. *cj1430c*) and *kpsM* (a.k.a. *cj1448c*) genes were amplified via Polymerase Chain Reaction (PCR) from chromosomal DNA of *C. jejuni* strain ATCC 700819 using primers *cj1427c* P2/P3, *cj1428c* P2/P3, *cj1430c* P2/P3, and *kpsM* P2/P3 (Table 4). The PCR was performed using Expand Long Range DNA polymerase (Roche) according to Manufacturer's instructions. For *wcaG*, *mlghB* and *mlghC*, the PCR products were cut with BamHI and NcoI and were ligated to a pET23 vector derivative (Newton and Mangroo, 1999) that had been cut with the same enzymes. The *kpsM* gene was cloned into the EcoRV site of pBluescript KS(+), following blunting of the PCR product by T4 DNA Polymerase (Roche, Canada). After transformation of ligation reactions into *E. coli* DH5α and selection on ampicillin, the resulting plasmids (pET/*wcaG*, pET/*mlghC*, pET/*mlghB* and pBluescriptKS/*kpsM*) were extracted and checked by DNA sequencing (Robarts Research Institute, London, Ontario).

Disruption constructs were generated by performing inverse PCR on pET/*wcaG*, pET/*mlghC*, pET/*mlghB* using primers *cj1427c* P4/P5, *cj1428c* P4/P5, *cj1430c* P4/P5, which contain KpnI and ApaI sites. The chloramphenicol resistance cassette (*cat*) was amplified from plasmid pRY111 (kindly provided by P. Guerry, Naval Medical Research Center, MD, USA) using primers CatColi P2 and CatColi P3 which contained ApaI and



KpnI sites, respectively. The amplicon was subsequently ligated into the inverse PCR reaction products following ApaI and KpnI digestion. The constructs were introduced into *E. coli* DH5 $\alpha$  and transformants were selected on ampicillin and chloramphenicol. The resulting pET/*wcaG::cat*, pET/*mlghC::cat*, and pET/*mlghB::cat*, constructs were extracted and checked by DNA sequencing. For the disruption of *kpsM*, inverse PCR was used to amplify pBluescriptKS/*kpsM* with the primers *kpsM* P4/P6 that contain BglII and NcoI sites, respectively. The kanamycin resistance cassette (*kan*) was amplified from plasmid pHel3 (Heuermann and Haas, 1998) using Aph3P1 and Aph3P2, which also contained BglII and NcoI restriction sites. The *kan* cassette was subsequently ligated into the pBluescriptKS/*kpsM* inverse PCR reaction product after BglII/NcoI digestion. Following *E. coli* DH5 $\alpha$  transformation, transformants were selected on ampicillin and kanamycin. The resulting pBluescriptKS/*kpsM::kan* construct was checked by DNA sequencing.

#### Generation of knockout mutants in *C. jejuni* ATCC 700819

The *wcaG::cat*, *mlghB::cat*, *mlghC::cat*, and *kpsM::kan* fragments were amplified using gene specific primers P2 and P3 (Table 4) containing NcoI and BamHI restriction sites respectively. *C. jejuni* chromosomal DNA digested with the same restriction enzymes was ligated to either end of the PCR fragments. The DNA was introduced into *C. jejuni* via natural transformation using 0.03% saponin (Nuijten *et al.*, 1989; Vijayakumar *et al.*, 2006). Potential transformants were selected on MH agar containing 15  $\mu$ g/ml chloramphenicol or 30  $\mu$ g/ml kanamycin, The transformants were checked by PCR using

gene specific and antibiotic cassette specific primers, as well as by Southern blotting using digoxigenin (Roche) labeled DNA probes.

As an alternative to obtain the *wcaG* mutant, the pET/*wcaG::cat* was methylated *in vitro* as described by others (Donahue *et al.*, 2000). Ten µg of plasmid DNA were treated with a cell-free extract of *C. jejuni* (containing 300-400 µg of proteins) in the presence of 200 nM S-adenosyl methionine (Sigma Aldrich). Methylation was carried out at 37°C for 1 hour in “methylation” buffer (20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 5 mM Na<sub>2</sub>EDTA, 1 mM dithiothreitol (DTT)) in a total volume of 200 µl. To obtain a cell-free extract, wild type *C. jejuni* was harvested from five TSA plates and resuspended in 4 ml of “methylation” buffer supplemented with protease inhibitor cocktail (Roche). This was passed through a French pressure cell press (Thermo Scientific) five times. The amount of protein in the cell-free extract was quantitated by Bradford Assay (Bradford, 1976). Following methylation, phenol:chloroform:isoamyl alcohol (in a 25:24:1 v:v:v ratio) was used to purify the DNA which was concentrated by sodium acetate/ethanol precipitation. The plasmid was introduced in *C. jejuni* by natural transformation as above, using 70 µl of cells resuspended at 10E9 cells/ml and 2 µg of DNA in a total volume of 80 µl, with recovery of ~ 8-10 h prior to selection on chloramphenicol.

#### *Preparation of complemented strains*

Complementation was performed by chromosomal integration in the 16S - 23S rRNA region as reported previously (Karlyshev and Wren, 2005). All primers used are listed in

Supplementary Table 1. The 16S – 23S rRNA region was amplified from genomic DNA using primers 16SrRNATop and 23SrRNABottom and was inserted into the KpnI/NotI sites of pBluescript KS (+). Clones were obtained using *E. coli* DH5 $\alpha$  and selection with ampicillin. Separately, the primer overlap extension method (Heckman and Pease, 2007) was used to fuse each gene of interest to the *ompE* promoter and to a *kan* resistance cassette. The promoter was amplified using primer ompEFor combined with ompERev1427, ompERev1428, or ompERev1430. The genes to be complemented were amplified using primer pairs 1427ForOmpE / 1427RevKan, 1428ForOmpE / 1428RevKan, or 1430ForOmpE / 1430RevKan. The *kan* cassette was amplified using primer AphP3 combined with KanFor1427, KanFor1428 or KanFor1430. All fragments were fused together by PCR using standard conditions. The fusions were then inserted into the XbaI site of the 16S–23SrRNA-containing vector from above to generate the final complementation constructs. Clones were selected with ampicillin and kanamycin and sequenced (Robarts Sequencing Facility, London, Ontario). The constructs were transformed into their respective mutants following *in vitro* methylation as reported above. Clones were selected on ampicillin and kanamycin and analysed by PCR to check integration of the constructs.

#### *Real-time PCR analyses*

Messenger RNA was extracted from *C. jejuni* using the GE-Healthcare mRNA midiprep kit, and cDNA synthesis was performed using the Biorad cDNA biosynthesis kit and random hexanucleotides following Manufacturer's instructions. Real-time PCR was performed on a Rotor-Gene 6000 (Corbett Life Science, Canada) using SYBR green

Supermix (Biorad) and gene-specific primers (Table 4). The optimal annealing temperature and primer concentrations were determined for each gene. The primer efficiencies were determined from standard curves established using serial dilutions of genomic DNA under optimal annealing temperature and primer concentrations. Negative controls for the reverse transcription did not contain the reverse transcriptase, while negative controls for the real time PCR did not contain template cDNA. The relative gene expression for each gene was calculated using the Pfaffl equation (Pfaffl, 2001):

$$\text{Ratio} = (E_{\text{target}})^{\Delta C_T^{\text{target(WT-mutant)}}} / (E_{\text{reference}})^{\Delta C_T^{\text{ref(WT-mutant)}}}$$

In this equation, target refers to the gene being studied and the reference is the housekeeping *cj1537c* gene, which encodes acetyl-CoA synthase (Parkhill *et al.*, 2000).  $E$  is the primer efficiency, and  $C_T$  corresponds to the number of PCR cycles necessary to observe a fluorescence signal above a set threshold.

#### *SDS-PAGE and Western blotting analysis of CPS*

Total surface carbohydrates (CPS and LOS) were extracted via SDS solubilization of *C. jejuni* cells (Hitchcock and Brown, 1983), followed by digestion of proteins by proteinase K treatment. They were separated on a 15% SDS-PAGE 5 cm mini gel and analyzed by Western blotting using Penner serotyping antibody HS:2 (kindly provided by Dr. M. Karmali, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario) since strain ATCC 700819 belongs to the HS:2 Penner serotype (Karmali *et al.*, 1983; Penner *et al.*, 1983) and CPS contributes to serotype specificity (Karlyshev *et al.*, 2000). Detection was performed with a fluorescently labeled anti-rabbit secondary

antibody (IRDye 680 Goat Anti-Rabbit, Licor Biosciences) on an Odyssey Infrared Imaging system (Licor Bioscience). Alternatively, CPS was extracted by hot water phenol (see below), ran on a 13 cm long gel and detection was performed by silver staining (Fomsgaard *et al.*, 1990).

780

781 *CPS isolation by the hot water phenol method*

782

*C. jejuni* ATCC 700819 wild type and mutants were grown on one hundred TSA plates for 24 hours and harvested in saline. The cells were then inoculated into 10 L of Brucella broth, containing 7.5% heat inactivated horse serum and 25 mM sodium pyruvate to an OD<sub>600nm</sub> of approximately 0.05. After 24 hours of growth (OD<sub>600nm</sub> approximately 0.3), the bacteria were spun down at 4200 × g (Avanti J-25I, Beckman-Coulter) for 30 min and the pellets were lyophilized. Purification of CPS was performed by hot water/phenol extraction (Westphal and Jann, 1965). Briefly, 2 g of dry cell pellet were re-suspended in 20 ml of MilliQ water pre-heated to 68°C. An equal amount of phenol (Fisher) preheated to 68°C was added to the pellet and sealed in a 50 ml conical tube. The samples were incubated at 68°C for 10 min with rapid stirring. They were allowed to cool to 10°C on ice and centrifuged for 30 min at 6300 × g (Eppendorf 5810R) and 10°C. The aqueous (top) phase was collected, and an equal amount of water was added to the remaining organic phase. The procedure was repeated three times and the aqueous phases were pooled. The aqueous phases were dialyzed (molecular weight cut off 12-14000 Da) against running water for 2-3 days until no phenol remained. The samples were lyophilized and re-suspended in double distilled water. Ultracentrifugation of the samples

for 30 hours at 4°C and  $110000 \times g$  (Optima Max-XP Ultracentrifuge, Beckman-Coulter) pelleted most of the LOS, while the CPS remained in the supernatant. The samples were lyophilized and re-suspended in 500  $\mu$ l double distilled water. Treatment with 200 mg of Proteinase K was carried out for 2 hours at 60°C to degrade any remaining proteins. The CPS samples were stored at -20°C until further required.

#### *Sugar analysis*

CPS samples (0.2 mg each) were hydrolyzed with 2 M  $\text{CF}_3\text{CO}_2\text{H}$  (120 °C, 2 h), and the monosaccharides were identified using a Biotronik LC-2000 sugar analyzer (Germany) on a column (15  $\times$  0.4 cm) of a Dionex A $\times$ 8-11 anion-exchange resin and Ostion LC AN B cation-exchange resin (7.5 cm column each). Sugars were eluted with a stepwise gradient of 0.17 M (20 min) and 0.3 M sodium borate buffer pH 8.0 at 0.5 ml min<sup>-1</sup> and detected with the bicinchoninate reagent.

#### *Nuclear magnetic resonance spectroscopy*

Samples were deuterium-exchanged by freeze-drying twice from 99.9% D<sub>2</sub>O and then examined as solutions in 99.95% D<sub>2</sub>O at 30° C on NMR spectra were recorded on an Avance II 600 spectrometer (Bruker, Germany) using internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d<sub>4</sub> ( $\delta_{\text{H}}$  0,  $\delta_{\text{C}}$  -1.6) or 85% H<sub>3</sub>PO<sub>4</sub> ( $\delta_{\text{P}}$  0.0) as references for calibration. The <sup>1</sup>H and <sup>13</sup>C NMR spectra were assigned partially using two-dimensional <sup>1</sup>H, <sup>1</sup>H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY),

<sup>1</sup>H, <sup>13</sup>C heteronuclear single-quantum coherence (HSQC), and <sup>1</sup>H, <sup>31</sup>P heteronuclear multiple-bond correlation (HMBC) experiments, which were performed using standard Bruker software. A mixing time of 100 ms was used in the TOCSY and ROESY experiments, and a 65-ms delay was used for evolution of long-range coupling in the <sup>1</sup>H, <sup>31</sup>P HMBC experiment. TopSpin 2.1 (Bruker) program was used to acquire and process the NMR data.

#### *Growth curves assays*

A 100 ml sidearm flask containing 20 ml MH broth and background antibiotics (pre-saturated with 85% N<sub>2</sub>, 10% CO<sub>2</sub>, and 5% O<sub>2</sub>) was inoculated with *C. jejuni* cells grown on TSA at a starting OD<sub>600nm</sub> of 0.075. The flask was sealed and incubated with shaking (120 rpm) at 37°C for up to 48 h. Growth was monitored over time using a Klett Summerson photocolimeter.

#### *Motility assays*

*C. jejuni* was harvested in MH broth and adjusted to OD<sub>600nm</sub> of 1.0 or 2.0. Motility plates (0.3% agar in MH) were stabbed in triplicate with the wild type or mutants and incubated for 24 h under microaerobic conditions at 37°C. The diameter of the motility halo was monitored over time.

#### *Bile salts susceptibility assay*

846

847 Following growth on TSA as described above, *C. jejuni* was harvested and washed once  
848 in saline and re-suspended in saline to an OD<sub>600nm</sub> of 0.1. Bile salts (50% cholate and  
849 50% deoxycholate, Sigma Aldrich) were diluted to the appropriate concentrations (0-2  
850 g/l) and 90 µl were aliquoted into the wells of a 96 well plate. Ten µl of the bacterial  
851 suspension were added to each well and incubated in microaerobic conditions at 37°C for  
852 15 minutes. The samples were washed once in TSB media and serially diluted for CFU  
853 counts.

854

#### 855 *SDS susceptibility assays*

856

857 For SDS sensitivity assays, different concentrations (0.002 to 0.03%) of SDS were  
858 prepared in TSB (supplemented with vancomycin and trimethoprim). A volume of 170  
859 µL of each SDS concentration was added to the wells of a 96 well plate. *C. jejuni* wild  
860 type and mutants were grown as previously described and re-suspended in TSB to an  
861 OD<sub>600 nm</sub> 1.0. To each well, 30 µl of bacterial suspension was added, and control wells  
862 were set up which contained no SDS. The plates were incubated at 37°C with shaking at  
863 180 rpm in microaerobic conditions for 15 h, at which point the OD<sub>600nm</sub> was read. The  
864 data were normalized to controls which did not receive any SDS treatment.

865

#### 866 *Serum susceptibility*

867



868 Fresh rabbit blood obtained from two rabbits was allowed to clot at room temperature.  
869 The serum was separated from the clot and centrifuged for 15 minutes at 10,000 rpm at  
870 4°C to remove remaining blood cells. Half of the serum was inactivated by incubation at  
871 56°C for 1 hr. Bacteria grown for 16-20 h on TSA were washed in saline and re-  
872 suspended to an OD<sub>600nm</sub> of 0.1. Serum (inactivated or not) was re-suspended in saline to  
873 the appropriate concentration (0% - 100%) and 90 µl were aliquoted into the wells of a 96  
874 well plate. To the wells, 10 µl of bacteria were added. The plates were then incubated  
875 with shaking (100 rpm) for 1.5 h in microaerobic conditions at 37°C. Following  
876 incubation, 100 µl TSB was added to the wells and the samples were serially diluted and  
877 plated for CFU counts. For time course experiments, bacterial samples were exposed to  
878 20% pooled rabbit serum over 1.5 h.

879

880 *Phagocytosis and survival within macrophages*

881

882 RAW 267.4 murine macrophages (obtained from ATCC) were grown in Dulbecco's  
883 Modified Eagles Medium (DMEM) containing 10% heat inactivated FBS, and passaged  
884 every 3-4 days to a maximum of 5 passages. Macrophages were seeded at  $\sim 2 \times 10^5$  cells  
885 per well in 6-well plates, and incubated overnight in DMEM (containing 10% inactivated  
886 FBS). They were counted (final counts about  $10^6$  per well after incubation) and *C. jejuni*  
887 bacteria that had been resuspended in tryptic soy (TS) broth and adjusted at the  
888 appropriate OD were added at a multiplicity of infection (MOI) of 1:100. After addition  
889 of the bacteria, the media composition was 97% (DMEM with 10% FBS) and 3% TS  
890 broth. The plates were centrifuged for 1 min at 300 x g to synchronize bacteria /

macrophage interaction. For adhesion experiments, the plates were incubated at 4°C for 30 minutes to promote adhesion in the absence of phagocytosis (Peterson *et al.*, 1977), washed with cold PBS five times and lysed in water. Control experiments showed that all strains resisted the water treatment (data not shown). The bacteria were then serially diluted and plated for CFU counts. For the time course of intracellular survival, the macrophages were exposed to the bacteria for 2 h at 37°C in a CO<sub>2</sub> incubator. The cells were then washed three times with PBS and incubated with DMEM containing 225 µg/ml gentamicin for 1 h to kill extracellular bacteria. The macrophages were then washed and incubated in DMEM for up to 3 h. At each time point, the macrophages were washed three times with PBS and lysed in water (20 min). The samples were serially diluted and plated for CFU counts. For infection time course experiments, the macrophages were exposed to the bacteria for various lengths of time. The macrophages were washed in PBS, treated with gentamicin, and lysed in water, as described above. The surviving bacteria were enumerated by CFU counting.

#### *Adhesion and invasion of wild type and mutants to Caco-2 cells*

Caco-2 cells (obtained from ATCC) were grown for 3 days in DMEM containing 25 mM glucose and supplemented with 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin until they formed a confluent monolayer (approximately 650,000 cells per well in 24-well plates) and were fully differentiated. The differentiation state of the cells was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate in a standard colorimetric assay

(detection at 405 nm) which reflects the production of alkaline phosphatase and is a hallmark of differentiation (Ferruzza *et al.*, 2012). The cells were infected for 5 h with *C. jejuni* at an MOI of 1:100. The bacteria were prepared as indicated for the macrophage experiments. The plates were centrifuged briefly (500 x g for 5 min at room temperature) to maximize contact between the bacteria and the cell monolayer. To determine total bacterial cell association (adhering and internalized bacteria), Caco-2 cell monolayers were washed 3 times, lysed with 0.1 % Triton X-100 for 10 min and CFUs were determined by plating serial dilutions. To determine the number of internalized bacteria, the infected monolayers were treated with 200 µg/ml gentamicin for 2 h to kill extracellular bacteria. The cells were then washed and treated as above to determine bacterial viable counts.

#### *Autoagglutination assay*

The autoagglutination assay was performed as previously described (Misawa and Blaser, 2000). Briefly, 2 ml of sterile PBS were inoculated with *C. jejuni* at an OD<sub>600nm</sub> of 1.0. At time points 0, 1 and 2 h, the top 1 ml from each tube was carefully removed and the optical density was read at 600 nm. Live images were acquired using an Axioscope 2 (Carl Zeiss) microscope with a 100× oil immersion objective coupled to a Qimaging (Burnaby) cooled charged-coupled device camera.

#### *Electron microscopy (EM)*

EM was performed at the EM facility of the department of Microbiology and Immunology led by Dr. S. Koval with uranyl acetate staining as previously described (Merkx-Jacques *et al.*, 2004).

#### *Chicken colonization assays*

Chicken colonization assays were performed as previously described (Vijayakumar *et al.*, 2006). *C. jejuni* was grown on TSA agar containing 5% sheep blood under microaerobic conditions at 42°C for 24 hours. The bacteria were re-suspended in PBS pH 7.4, and adjusted to an OD<sub>600nm</sub> of 0.35 (approx. 10E9 CFU/ml) and diluted 1:10 in PBS. Two-day-old white-leghorn specific pathogen-free chicks were orally administered 100 µl (10E7 CFU) of either the *C. jejuni* wild type or one of the mutants. Five days later, the chicks were euthanized, the caeca and their contents were harvested, weighed and homogenized. Viable counts were obtained from serial dilutions of samples that were plated on Campylobacter selective medium (CSM) plates (Quélab Inc., Montreal) for 48 hours.

#### **ACKNOWLEDGEMENTS**

We thank former lab members J. Griffin, A. Merks-Jacques and Dr. D. Ratnayake for advice on genetic manipulation of *C. jejuni* and on tissue culture, X. T. Bui for help with cloning and strain construction, N. Zebian for discussions on CPS purification and statistics, Dr. A. S. Shashkov (Zelinsky Institute, Russia) for help with NMR spectroscopy, Dr. P. Guerry (Naval Medical Research Center) for providing the pRYIII

vector, A. Nazli, J. Lu (McMaster University, Canada) and S. Kim (University of Western Ontario, Canada) for providing Caco-2 cells and advice for tissue culture experiments, Dr. M. Karmali (Laboratory for Foodborne Zoonoses Health Canada) for providing the *C. jejuni* serotype specific antibody, and Dr. J. Lam (Guelph University) for providing the anti lipid A antibody.

This work was supported primarily by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada to Dr. Creuzenet (RGPIN 240762-2001) and Dr. Dozois (RGPIN 250129-07) and by a grant from the Canadian Institutes of Health Research (CIHR) to Drs Creuzenet and Valvano (MOP119299)

Dr. Creuzenet was the recipient of a University Faculty Award from NSERC and a Premier's Research Excellence Award (Ontario, Canada). Dr. C.M. Dozois was supported by funds from the Canada Research Chair in Bacterial Infectious Diseases. Dr Valvano was a Canada Research Chair in Infectious Diseases and Microbial Pathogenesis.

## REFERENCES:

- Ashgar, S.S., Oldfield, N.J., Wooldridge, K.G., Jones, M.A., Irving, G.J., Turner, D.P., and Ala'Aldeen, D.A. (2007) CapA, an autotransporter protein of *Campylobacter jejuni*, mediates association with human epithelial cells and colonization of the chicken gut. *J Bacteriol* **189**: 1856-1865.
- Aspinall, G.O., McDonald, A.G., and Pang, H. (1992) Structures of the O chains from lipopolysaccharides of *Campylobacter jejuni* serotypes O:23 and O:36. *Carbohydr Res* **231**: 13-30.
- Aspinall, G.O., McDonald, A.G., Pang, H., Kurjanczyk, L.A., and Penner, J.L. (1993) An antigenic polysaccharide from *Campylobacter coli* serotype O:30. Structure of a teichoic acid-like antigenic polysaccharide associated with the lipopolysaccharide. *J Biol Chem* **268**: 18321-18329.
- Aspinall, G.O., Lynch, C.M., Pang, H., Shaver, R.T., and Moran, A.P. (1995a) Chemical structures of the core region of *Campylobacter jejuni* O:3 lipopolysaccharide and an associated polysaccharide. *Eur J Biochem* **231**: 570-578.
- Aspinall, G.O., Monteiro, M.A., Pang, H., Kurjanczyk, L.A., and Penner, J.L. (1995b) Lipo-oligosaccharide of *Campylobacter lari* strain PC 637. Structure of the liberated oligosaccharide and an associated extracellular polysaccharide. *Carbohydr Res* **279**: 227-244.
- Bachtiar, B.M., Coloe, P.J., and Fry, B.N. (2007) Knockout mutagenesis of the *kpsE* gene of *Campylobacter jejuni* 81116 and its involvement in bacterium-host interactions. *FEMS Immunol Med Microbiol* **49**: 149-154.
- Bacon, D.J., Szymanski, C.M., Burr, D.H., Silver, R.P., Alm, R.A., and Guerry, P. (2001) A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol Microbiol* **40**: 769-777.
- Beery, J.T., Hugdahl, M.B., and Doyle, M.P. (1988) Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl Environ Microbiol* **54**: 2365-2370.
- Begley, M., Gahan, C.G., and Hill, C. (2002) Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. *Appl Environ Microbiol* **68**: 6005-6012.
- Benz, I., and Schmidt, M.A. (2001) Glycosylation with heptose residues mediated by the aah gene product is essential for adherence of the AIDA-I adhesin. *Mol Microbiol* **40**: 1403-1413.
- Biswas, D., Itoh, K., and Sasakawa, C. (2000) Uptake pathways of clinical and healthy animal isolates of *Campylobacter jejuni* into INT-407 cells. *FEMS Immunol Med Microbiol* **29**: 203-211.
- Biswas, D., Fernando, U.M., Reiman, C.D., Willson, P.J., Townsend, H.G., Potter, A.A., and Allan, B.J. (2007) Correlation between in vitro secretion of virulence-associated proteins of *Campylobacter jejuni* and colonization of chickens. *Curr Microbiol* **54**: 207-212.
- Black, R., Levine, M., Clements, M., Hughes, T., and Blaser, M. (1988) Experimental *Campylobacter* infections in humans. *J. Infect. Dis.* **157**: 472-478.

- 1019 Boulnois, G.J., Roberts, I.S., Hodge, R., Hardy, K.R., Jann, K.B., and Timmis, K.N.  
 1020 (1987) Analysis of the K1 capsule biosynthesis genes of *Escherichia coli*:  
 1021 definition of three functional regions for capsule production. *Mol Gen Genet* **208**:  
 1022 242-246.
- 1023 Boulnois, G.J., and Roberts, I.S. (1990) Genetics of capsular polysaccharide production  
 1024 in bacteria. *Curr Top Microbiol Immunol* **150**: 1-18.
- 1025 Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram  
 1026 quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*  
 1027 **72**: 248-254.
- 1028 Butty, F.D., Aucoin, M., Morrison, L., Ho, N., Shaw, G.S., and Creuzenet, C. (2009)  
 1029 Elucidating the formation of 6-deoxyheptose: biochemical characterization of the  
 1030 GDP-D-glycero-D-manno-heptose C6 dehydratase, DmhA and its associated C4  
 1031 reductase, DmhB. *Biochemistry* **48**: 7764-7775.
- 1032 Campbell, N.B., Ruaux, C.G., Shifflett, D.E., Steiner, J.M., Williams, D.A., and  
 1033 Blikslager, A.T. (2004) Physiological concentrations of bile salts inhibit recovery  
 1034 of ischemic-injured porcine ileum. *Am J Physiol Gastrointest Liver Physiol* **287**:  
 1035 G399-407.
- 1036 Carrillo, C.D., Taboada, E., Nash, J.H., Lanthier, P., Kelly, J., Lau, P.C., Verhulp, R.,  
 1037 Mykytczuk, O., Sy, J., Findlay, W.A., Amoako, K., Gomis, S., Willson, P.,  
 1038 Austin, J.W., Potter, A., Babiuk, L., Allan, B., and Szymanski, C.M. (2004)  
 1039 Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals  
 1040 coordinate regulation of motility and virulence by *flhA*. *J Biol Chem* **279**: 20327-  
 1041 20338.
- 1042 Champion, O.L., Karlyshev, A.V., Senior, N.J., Woodward, M., La Ragione, R., Howard,  
 1043 S.L., Wren, B.W., and Titball, R.W. (2010) Insect infection model for  
 1044 *Campylobacter jejuni* reveals that O-methyl phosphoramidate has insecticidal  
 1045 activity. *J Infect Dis* **201**: 776-782.
- 1046 Chen, Y.H., Poly, F., Pakulski, Z., Guerry, P., and Monteiro, M.A. (2008) The chemical  
 1047 structure and genetic locus of *Campylobacter jejuni* CG8486 (serotype HS:4)  
 1048 capsular polysaccharide: the identification of 6-deoxy-D-ido-heptopyranose.  
 1049 *Carbohydr Res* **343**: 1034-1040.
- 1050 Corcionivoschi, N., Clyne, M., Lyons, A., Elmi, A., Gundogdu, O., Wren, B.W., Dorrell,  
 1051 N., Karlyshev, A.V., and Bourke, B. (2009) *Campylobacter jejuni* cocultured with  
 1052 epithelial cells reduces surface capsular polysaccharide expression. *Infect Immun*  
 1053 **77**: 1959-1967.
- 1054 Corcoran, A.T., Annuk, H., and Moran, A.P. (2006) The structure of the lipid anchor of  
 1055 *Campylobacter jejuni* polysaccharide. *FEMS Microbiol Lett* **257**: 228-235.
- 1056 Cortes, G., Borrell, N., de Astorza, B., Gomez, C., Sauleda, J., and Alberti, S. (2002)  
 1057 Molecular analysis of the contribution of the capsular polysaccharide and the  
 1058 lipopolysaccharide O side chain to the virulence of *Klebsiella pneumoniae* in a  
 1059 murine model of pneumonia. *Infect Immun* **70**: 2583-2590.
- 1060 Cuthbertson, L., Kos, V., and Whitfield, C. (2010) ABC transporters involved in export  
 1061 of cell surface glycoconjugates. *Microbiol Mol Biol Rev* **74**: 341-362.
- 1062 Day, W.A., Jr., Sajecki, J.L., Pitts, T.M., and Joens, L.A. (2000) Role of catalase in  
 1063 *Campylobacter jejuni* intracellular survival. *Infect Immun* **68**: 6337-6345.



- 1064 De Melo, M.A., Gabbiani, G., and Pechere, J.C. (1989) Cellular events and intracellular  
1065 survival of *Campylobacter jejuni* during infection of HEp-2 cells. *Infect Immun*  
1066 **57**: 2214-2222.
- 1067 Donahue, J.P., Israel, D.A., Peek, R.M., Blaser, M.J., and Miller, G.G. (2000)  
1068 Overcoming the restriction barrier to plasmid transformation of *Helicobacter*  
1069 *pylori*. *Mol Microbiol* **37**: 1066-1074.
- 1070 Dugar, G., Herbig, A., Forstner, K.U., Heidrich, N., Reinhardt, R., Nieselt, K., and  
1071 Sharma, C.M. (2013) High-resolution transcriptome maps reveal strain-specific  
1072 regulatory features of multiple *Campylobacter jejuni* isolates. *PLoS Genet* **9**:  
1073 e1003495.
- 1074 Everest, P.H., Goossens, H., Butzler, J.P., Lloyd, D., Knutton, S., Ketley, J.M., and  
1075 Williams, P.H. (1992) Differentiated Caco-2 cells as a model for enteric invasion  
1076 by *Campylobacter jejuni* and *C. coli*. *J Med Microbiol* **37**: 319-325.
- 1077 Fauchere, J.L., Rosenau, A., Veron, M., Moyon, E.N., Richard, S., and Pfister, A. (1986)  
1078 Association with HeLa cells of *Campylobacter jejuni* and *Campylobacter coli*  
1079 isolated from human feces. *Infect Immun* **54**: 283-287.
- 1080 Ferruzza, S., Rossi, C., Scarino, M.L., and Sambuy, Y. (2012) A protocol for in situ  
1081 enzyme assays to assess the differentiation of human intestinal Caco-2 cells.  
1082 *Toxicol In Vitro* **26**: 1247-1251.
- 1083 Fomsgaard, A., Freudenberg, M.A., and Galanos, C. (1990) Modification of the silver  
1084 staining technique to detect lipopolysaccharide in polyacrylamide gels. *J Clin*  
1085 *Microbiol* **28**: 2627-2631.
- 1086 Ganan, M., Campos, G., Munoz, R., Carrascosa, A.V., de Pascual-Teresa, S., and  
1087 Martinez-Rodriguez, A.J. (2010) Effect of growth phase on the adherence to and  
1088 invasion of Caco-2 epithelial cells by *Campylobacter*. *Int J Food Microbiol* **140**:  
1089 14-18.
- 1090 Gerritsen van der Hoop, A., and Veringa, E.M. (1993) Cholecystitis caused by  
1091 *Campylobacter jejuni*. *Clin Infect Dis* **17**: 133.
- 1092 Godschalk, P.C., Heikema, A.P., Gilbert, M., Komagamine, T., Ang, C.W., Glerum, J.,  
1093 Brochu, D., Li, J., Yuki, N., Jacobs, B.C., van Belkum, A., and Endtz, H.P. (2004)  
1094 The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody  
1095 induction in Guillain-Barre syndrome. *J Clin Invest* **114**: 1659-1665.
- 1096 Godschalk, P.C., Kuijf, M.L., Li, J., St Michael, F., Ang, C.W., Jacobs, B.C., Karwaski,  
1097 M.F., Brochu, D., Moterassed, A., Endtz, H.P., van Belkum, A., and Gilbert, M.  
1098 (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide  
1099 outer cores associated with Guillain-Barre and Miller Fisher syndromes. *Infect*  
1100 *Immun* **75**: 1245-1254.
- 1101 Guerry, P., Ewing, C.P., Hickey, T.E., Prendergast, M.M., and Moran, A.P. (2000)  
1102 Sialylation of lipooligosaccharide cores affects immunogenicity and serum  
1103 resistance of *Campylobacter jejuni*. *Infect Immun* **68**: 6656-6662.
- 1104 Guerry, P., Szymanski, C.M., Prendergast, M.M., Hickey, T.E., Ewing, C.P., Pattarini,  
1105 D.L., and Moran, A.P. (2002) Phase Variation of *Campylobacter jejuni* 81-176  
1106 Lipooligosaccharide Affects Ganglioside Mimicry and Invasiveness In Vitro.  
1107 *Infect Immun* **70**: 787-793.



- Guerry, P., Ewing, C.P., Schirm, M., Lorenzo, M., Kelly, J., Pattarini, D., Majam, G., Thibault, P., and Logan, S. (2006) Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence. *Mol Microbiol* **60**: 299-311.
- Hashimoto, Y., Li, N., Yokoyama, H., and Ezaki, T. (1993) Complete nucleotide sequence and molecular characterization of *ViaB* region encoding Vi antigen in *Salmonella typhi*. *J Bacteriol* **175**: 4456-4465.
- Heckman, K.L., and Pease, L.R. (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc* **2**: 924-932.
- Heuermann, D., and Haas, R. (1998) A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. *Mol Gen Genet* **257**: 519-528.
- Hitchcock, P.J., and Brown, T.M. (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* **154**: 269-277.
- Ho, N., Kondakova, A.N., Knirel, Y.A., and Creuzenet, C. (2008) The biosynthesis and biological role of 6-deoxyheptose in the lipopolysaccharide O-antigen of *Yersinia pseudotuberculosis*. *Mol Microbiol* **68**: 424-447.
- Hsieh, Y.C., Liang, S.M., Tsai, W.L., Chen, Y.H., Liu, T.Y., and Liang, C.M. (2003) Study of capsular polysaccharide from *Vibrio parahaemolyticus*. *Infect Immun* **71**: 3329-3336.
- Jann, K., Dengler, T., and Jann, B. (1992) Core-lipid A on the K40 polysaccharide of *Escherichia coli* O8:K40:H9, a representative of group I capsular polysaccharides. *Zentralbl Bakteriol* **276**: 196-204.
- Jin, S., Joe, A., Lynett, J., Hani, E.K., Sherman, P., and Chan, V.L. (2001) JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. *Mol Microbiol* **39**: 1225-1236.
- Jones, M.A., Marston, K.L., Woodall, C.A., Maskell, D.J., Linton, D., Karlyshev, A.V., Dorrell, N., Wren, B.W., and Barrow, P.A. (2004) Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract. *Infect Immun* **72**: 3769-3776.
- Kakuda, T., and DiRita, V.J. (2006) Cj1496c encodes a *Campylobacter jejuni* glycoprotein that influences invasion of human epithelial cells and colonization of the chick gastrointestinal tract. *Infect Immun* **74**: 4715-4723.
- Karlyshev, A.V., Henderson, J., Ketley, J.M., and Wren, B.W. (1999) Procedure for the investigation of bacterial genomes: random shot-gun cloning, sample sequencing and mutagenesis of *Campylobacter jejuni*. *Biotechniques* **26**: 50-52, 54, 56.
- Karlyshev, A.V., Linton, D., Gregson, N.A., Lastovica, A.J., and Wren, B.W. (2000) Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity. *Mol Microbiol* **35**: 529-541.
- Karlyshev, A.V., McCrossan, M.V., and Wren, B.W. (2001) Demonstration of polysaccharide capsule in *Campylobacter jejuni* using electron microscopy. *Infect Immun* **69**: 5921-5924.
- Karlyshev, A.V., and Wren, B.W. (2001) Detection and initial characterization of novel capsular polysaccharide among diverse *Campylobacter jejuni* strains using alcian blue dye. *J Clin Microbiol* **39**: 279-284.

- 1154 Karlyshev, A.V., Everest, P., Linton, D., Cawthraw, S., Newell, D.G., and Wren, B.W.  
1155 (2004) The *Campylobacter jejuni* general glycosylation system is important for  
1156 attachment to human epithelial cells and in the colonization of chicks.  
1157 *Microbiology* **150**: 1957-1964.
- 1158 Karlyshev, A.V., Champion, O.L., Churcher, C., Brisson, J.R., Jarrell, H.C., Gilbert, M.,  
1159 Brochu, D., St Michael, F., Li, J., Wakarchuk, W.W., Goodhead, I., Sanders, M.,  
1160 Stevens, K., White, B., Parkhill, J., Wren, B.W., and Szymanski, C.M. (2005)  
1161 Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for  
1162 the generation of structural diversity and the ability to form complex heptoses.  
1163 *Mol Microbiol* **55**: 90-103.
- 1164 Karlyshev, A.V., and Wren, B.W. (2005) Development and application of an insertional  
1165 system for gene delivery and expression in *Campylobacter jejuni*. *Appl Environ*  
1166 *Microbiol* **71**: 4004-4013.
- 1167 Karmali, M.A., Penner, J.L., Fleming, P.C., Williams, A., and Hennessy, J.N. (1983) The  
1168 serotype and biotype distribution of clinical isolates of *Campylobacter jejuni* and  
1169 *Campylobacter coli* over a three-year period. *J Infect Dis* **147**: 243-246.
- 1170 Keo, T., Collins, J., Kunwar, P., Blaser, M.J., and Iovine, N.M. (2011) *Campylobacter*  
1171 capsule and lipooligosaccharide confer resistance to serum and cationic  
1172 antimicrobials. *Virulence* **2**: 30-40.
- 1173 Kiehlbauch, J.A., Albach, R.A., Baum, L.L., and Chang, K.P. (1985) Phagocytosis of  
1174 *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes.  
1175 *Infect Immun* **48**: 446-451.
- 1176 Kilcoyne, M., Moran, A.P., Shashkov, A.S., Senchenkova, S.N., Ferris, J.A., Corcoran,  
1177 A.T., and Savage, A.V. (2006) Molecular origin of two polysaccharides of  
1178 *Campylobacter jejuni* 81116. *FEMS Microbiol Lett* **263**: 214-222.
- 1179 Kneidinger, B., Graninger, M., Puchberger, M., Kosma, P., and Messner, P. (2001)  
1180 Biosynthesis of nucleotide-activated D-glycero-D-manno-heptose. *J Biol Chem*  
1181 **276**: 20935-20944.
- 1182 Kondakova, A.N., Ho, N., Bystrova, O.V., Shashkov, A.S., Lindner, B., Creuzenet, C.,  
1183 and Knirel, Y.A. (2008) Structural studies of the O-antigens of *Yersinia*  
1184 *pseudotuberculosis* O:2a and mutants thereof with impaired 6-deoxy-D-manno-  
1185 heptose biosynthesis pathway. *Carbohydr Res* **343**: 1383-1389.
- 1186 Konkel, M.E., Hayes, S.F., Joens, L.A., and Cieplak, W., Jr. (1992) Characteristics of the  
1187 internalization and intracellular survival of *Campylobacter jejuni* in human  
1188 epithelial cell cultures. *Microb Pathog* **13**: 357-370.
- 1189 Konkel, M.E., Kim, B.J., Rivera-Amill, V., and Garvis, S.G. (1999) Bacterial secreted  
1190 proteins are required for the internalization of *Campylobacter jejuni* into cultured  
1191 mammalian cells. *Mol Microbiol* **32**: 691-701.
- 1192 Konkel, M.E., Klena, J.D., Rivera-Amill, V., Monteville, M.R., Biswas, D., Raphael, B.,  
1193 and Mickelson, J. (2004) Secretion of virulence proteins from *Campylobacter*  
1194 *jejuni* is dependent on a functional flagellar export apparatus. *J Bacteriol* **186**:  
1195 3296-3303.
- 1196 Konkel, M.E., Christensen, J.E., Keech, A.M., Monteville, M.R., Klena, J.D., and Garvis,  
1197 S.G. (2005) Identification of a fibronectin-binding domain within the  
1198 *Campylobacter jejuni* CadF protein. *Mol Microbiol* **57**: 1022-1035.

- 1199 Kroll, J.S., Hopkins, I., and Moxon, E.R. (1988) Capsule loss in *H. influenzae* type b  
1200 occurs by recombination-mediated disruption of a gene essential for  
1201 polysaccharide export. *Cell* **53**: 347-356.
- 1202 Lee, A., O'Rourke, J.L., Barrington, P.J., and Trust, T.J. (1986) Mucus colonization as a  
1203 determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a  
1204 mouse cecal model. *Infect Immun* **51**: 536-546.
- 1205 Lin, J., Sahin, O., Michel, L.O., and Zhang, Q. (2003) Critical role of multidrug efflux  
1206 pump CmeABC in bile resistance and in vivo colonization of *Campylobacter*  
1207 *jejuni*. *Infect Immun* **71**: 4250-4259.
- 1208 Lin, J., Cagliero, C., Guo, B., Barton, Y.W., Maurel, M.C., Payot, S., and Zhang, Q.  
1209 (2005) Bile Salts Modulate Expression of the CmeABC Multidrug Efflux Pump  
1210 in *Campylobacter jejuni*. *J Bacteriol* **187**: 7417-7424.
- 1211 Manninen, K.I., Prescott, J.F., and Dohoo, I.R. (1982) Pathogenicity of *Campylobacter*  
1212 *jejuni* isolates from animals and humans. *Infect Immun* **38**: 46-52.
- 1213 Maue, A.C., Mohawk, K.L., Giles, D.K., Poly, F., Ewing, C.P., Jiao, Y., Lee, G., Ma, Z.,  
1214 Monteiro, M.A., Hill, C.L., Ferderber, J.S., Porter, C.K., Trent, M.S., and Guerry,  
1215 P. (2013) The polysaccharide capsule of *Campylobacter jejuni* modulates the host  
1216 immune response. *Infect Immun* **81**: 665-672.
- 1217 McCallum, M., Shaw, G.S., and Creuzenet, C. (2011) Characterization of the dehydratase  
1218 WcbK and the reductase WcaG involved in GDP-6-deoxy-manno-heptose  
1219 biosynthesis in *Campylobacter jejuni*. *Biochemical Journal* **439**: 235-248.
- 1220 McCallum, M., Shaw, S.D., Shaw, G.S., and Creuzenet, C. (2012) Complete 6-deoxy-D-  
1221 *altro*-heptose biosynthesis pathway from *Campylobacter jejuni*: more complex  
1222 than anticipated. *J Biol Chem* **287**: 29776-29788.
- 1223 McCallum, M., Shaw, G.S., and Creuzenet, C. (2013) Comparison of Predicted  
1224 Epimerases and Reductases of the *Campylobacter jejuni* D-*altro*- and L-*gluco*-  
1225 Heptose Synthesis Pathways. *J Biol Chem* **288**: 19569-19580.
- 1226 McNally, D.J., Lamoureux, M.P., Karlyshev, A.V., Fiori, L.M., Li, J., Thacker, G.,  
1227 Coleman, R.A., Khieu, N.H., Wren, B.W., Brisson, J.R., Jarrell, H.C., and  
1228 Szymanski, C.M. (2007) Commonality and biosynthesis of the O-methyl  
1229 phosphoramidate capsule modification in *Campylobacter jejuni*. *J Biol Chem* **282**:  
1230 28566-28576.
- 1231 Merckx-Jacques, A., Obhi, R.K., Bethune, G., and Creuzenet, C. (2004) The *Helicobacter*  
1232 *pylori* *flaA1* and *wbpB* genes control lipopolysaccharide and flagellum synthesis  
1233 and function. *J. Bacteriology* **186**: 2253-2265.
- 1234 Misawa, N., and Blaser, M.J. (2000) Detection and characterization of autoagglutination  
1235 activity by *Campylobacter jejuni*. *Infect Immun* **68**: 6168-6175.
- 1236 Mishu, B., and Blaser, M.J. (1993) Role of infection due to *Campylobacter jejuni* in the  
1237 initiation of Guillain-Barre syndrome. *Clin Infect Dis* **17**: 104-108.
- 1238 Mixter, P.F., Klena, J.D., Flom, G.A., Siegesmund, A.M., and Konkel, M.E. (2003) In  
1239 vivo tracking of *Campylobacter jejuni* by using a novel recombinant expressing  
1240 green fluorescent protein. *Appl Environ Microbiol* **69**: 2864-2874.
- 1241 Newell, D.G., and Pearson, A. (1984) The invasion of epithelial cell lines and the  
1242 intestinal epithelium of infant mice by *Campylobacter jejuni/coli*. *J Diarrhoeal*  
1243 *Dis Res* **2**: 19-26.

- 1244 Newell, D.G., McBride, H., Saunders, F., Dehele, Y., and Pearson, A.D. (1985) The  
1245 virulence of clinical and environmental isolates of *Campylobacter jejuni*. *J Hyg*  
1246 (*Lond*) **94**: 45-54.
- 1247 Newton, D.T., and Mangroo, D. (1999) Mapping the active site of the *Haemophilus*  
1248 *influenzae* methionyl-tRNA formyltransferase: residues important for catalysis  
1249 and tRNA binding. *Biochem J* **339**: 63-69.
- 1250 Nuijten, P.J., Bleumink-Pluym, N.M., Gaastra, W., and van der Zeijst, B.A. (1989)  
1251 Flagellin expression in *Campylobacter jejuni* is regulated at the transcriptional  
1252 level. *Infect Immun* **57**: 1084-1088.
- 1253 Pace, J.L., Chai, T.J., Rossi, H.A., and Jiang, X. (1997) Effect of bile on *Vibrio*  
1254 *parahaemolyticus*. *Appl Environ Microbiol* **63**: 2372-2377.
- 1255 Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D.,  
1256 Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev,  
1257 A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A.,  
1258 Rutherford, K.M., van Vliet, A.H., Whitehead, S., and Barrell, B.G. (2000) The  
1259 genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals  
1260 hypervariable sequences. *Nature* **403**: 665-668.
- 1261 Pavelka, M.S., Jr., Hayes, S.F., and Silver, R.P. (1994) Characterization of KpsT, the  
1262 ATP-binding component of the ABC-transporter involved with the export of  
1263 capsular polysialic acid in *Escherichia coli* K1. *J Biol Chem* **269**: 20149-20158.
- 1264 Pei, Z., and Blaser, M.J. (1993) PEB1, the major cell-binding factor of *Campylobacter*  
1265 *jejuni*, is a homolog of the binding component in gram-negative nutrient transport  
1266 systems. *J Biol Chem* **268**: 18717-18725.
- 1267 Penner, J.L., Hennessy, J.N., and Congi, R.V. (1983) Serotyping of *Campylobacter jejuni*  
1268 and *Campylobacter coli* on the basis of thermostable antigens. *Eur J Clin*  
1269 *Microbiol* **2**: 378-383.
- 1270 Perez de la Cruz Moreno, M., Oth, M., Deferme, S., Lammert, F., Tack, J., Dressman, J.,  
1271 and Augustijns, P. (2006) Characterization of fasted-state human intestinal fluids  
1272 collected from duodenum and jejunum. *J Pharm Pharmacol* **58**: 1079-1089.
- 1273 Perry, M.B., MacLean, L.L., Schollaardt, T., Bryan, L.E., and Ho, M. (1995) Structural  
1274 characterization of the lipopolysaccharide O antigens of *Burkholderia*  
1275 *pseudomallei*. *Infect Immun* **63**: 3348-3352.
- 1276 Peterson, P.K., Verhoef, J., and Quie, P.G. (1977) Influence of temperature on  
1277 opsonization and phagocytosis of *staphylococci*. *Infect Immun* **15**: 175-179.
- 1278 Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time  
1279 RT-PCR. *Nucleic Acids Res* **29**: e45.
- 1280 Preston, M.A., and Penner, J.L. (1989) Characterization of cross-reacting serotypes of  
1281 *Campylobacter jejuni*. *Can J Microbiol* **35**: 265-273.
- 1282 Rautelin, H., and Hanninen, M.L. (2000) *Campylobacters*: the most common bacterial  
1283 enteropathogens in the Nordic countries. *Ann Med* **32**: 440-445.
- 1284 Reckseidler-Zenteno, S.L., DeVinney, R., and Woods, D.E. (2005) The capsular  
1285 polysaccharide of *Burkholderia pseudomallei* contributes to survival in serum by  
1286 reducing complement factor C3b deposition. *Infect Immun* **73**: 1106-1115.
- 1287 Reckseidler, S.L., DeShazer, D., Sokol, P.A., and Woods, D.E. (2001) Detection of  
1288 bacterial virulence genes by subtractive hybridization: identification of capsular



- polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. *Infect Immun* **69**: 34-44.
- Roberts, I.S., Mountford, R., Hodge, R., Jann, K.B., and Boulnois, G.J. (1988a) Common organization of gene clusters for production of different capsular polysaccharides (K antigens) in *Escherichia coli*. *J Bacteriol* **170**: 1305-1310.
- Roberts, M., Roberts, I., Korhonen, T.K., Jann, K., Bitter-Suermann, D., Boulnois, G.J., and Williams, P.H. (1988b) DNA probes for K-antigen (capsule) typing of *Escherichia coli*. *J Clin Microbiol* **26**: 385-387.
- Rowe, S., Hodson, N., Griffiths, G., and Roberts, I.S. (2000) Regulation of the *Escherichia coli* K5 capsule gene cluster: evidence for the roles of H-NS, BipA, and integration host factor in regulation of group 2 capsule gene clusters in pathogenic *E. coli*. *J Bacteriol* **182**: 2741-2745.
- Salloway, S., Mermel, L.A., Seamans, M., Aspinall, G.O., Nam Shin, J.E., Kurjanczyk, L.A., and Penner, J.L. (1996) Miller-Fisher syndrome associated with *Campylobacter jejuni* bearing lipopolysaccharide molecules that mimic human ganglioside GD3. *Infect Immun* **64**: 2945-2949.
- Samuelsson, K., Lindberg, B., and Brubaker, R.R. (1974) Structure of O-specific side chains of lipopolysaccharides from *Yersinia pseudotuberculosis*. *J Bacteriol* **117**: 1010-1016.
- Silver, R.P., Prior, K., Nsahlai, C., and Wright, L.F. (2001) ABC transporters and the export of capsular polysaccharides from gram-negative bacteria. *Res Microbiol* **152**: 357-364.
- St Michael, F., Szymanski, C.M., Li, J., Chan, K.H., Khieu, N.H., Larocque, S., Wakarchuk, W.W., Brisson, J.R., and Monteiro, M.A. (2002) The structures of the lipooligosaccharide and capsule polysaccharide of *Campylobacter jejuni* genome sequenced strain NCTC 11168. *Eur J Biochem* **269**: 5119-5136.
- Sternberg, M.J., Tamaddoni-Nezhad, A., Lesk, V.I., Kay, E., Hitchen, P.G., Cootes, A., van Alphen, L.B., Lamoureux, M.P., Jarrell, H.C., Rawlings, C.J., Soo, E.C., Szymanski, C.M., Dell, A., Wren, B.W., and Muggleton, S.H. (2013) Gene Function Hypotheses for the *Campylobacter jejuni* Glycome Generated by a Logic-Based Approach. *J Mol Biol* **425**: 186-197.
- Stevens, M.P., Clarke, B.R., and Roberts, I.S. (1997) Regulation of the *Escherichia coli* K5 capsule gene cluster by transcription antitermination. *Mol Microbiol* **24**: 1001-1012.
- Szymanski, C.M., King, M., Haardt, M., and Armstrong, G.D. (1995) *Campylobacter jejuni* motility and invasion of Caco-2 cells. *Infect Immun* **63**: 4295-4300.
- Szymanski, C.M., Burr, D.H., and Guerry, P. (2002) *Campylobacter* protein glycosylation affects host cell interactions. *Infect Immun* **70**: 2242-2244.
- Szymanski, C.M., Michael, F.S., Jarrell, H.C., Li, J., Gilbert, M., Larocque, S., Vinogradov, E., and Brisson, J.R. (2003) Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from campylobacter cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. *J Biol Chem* **278**: 24509-24520.
- Udayakumar, D., and Sanaullah, M. (2009) *Campylobacter* cholecystitis. *Int J Med Sci* **6**: 374-375.

- 1334 Valvano, M.A., Messner, P., and Kosma, P. (2002) Novel pathways for biosynthesis of  
1335 nucleotide-activated *glycero-manno*-heptose precursors of bacterial glycoproteins  
1336 and cell surface polysaccharides. *Microbiology* **148**: 1979-1989.
- 1337 van Alphen, L.B., Wenzel, C.Q., Richards, M.R., Fodor, C., Ashmus, R.A., Stahl, M.,  
1338 Karlyshev, A.V., Wren, B.W., Stintzi, A., Miller, W.G., Lowary, T.L., and  
1339 Szymanski, C.M. (2014) Biological roles of the O-methyl phosphoramidate  
1340 capsule modification in *Campylobacter jejuni*. *PLoS One* **9**: e87051.
- 1341 van Sorge, N.M., Cole, J.N., Kuipers, K., Henningham, A., Aziz, R.K., Kasirer-Friede,  
1342 A., Lin, L., Berends, E.T., Davies, M.R., Dougan, G., Zhang, F., Dahesh, S.,  
1343 Shaw, L., Gin, J., Cunningham, M., Merriman, J.A., Hutter, J., Lepenies, B.,  
1344 Rooijackers, S.H., Malley, R., Walker, M.J., Shattil, S.J., Schlievert, P.M.,  
1345 Choudhury, B., and Nizet, V. (2014) The classical lancefield antigen of group a  
1346 *Streptococcus* is a virulence determinant with implications for vaccine design.  
1347 *Cell Host Microbe* **15**: 729-740.
- 1348 Vijayakumar, S., Merckx-Jacques, A., Ratnayake, D., Gryski, I., Obhi, R.K., Houle, S.,  
1349 Dozois, C., and Creuzenet, C. (2006) Cj1121c, a novel UDP-4-keto-6-deoxy-  
1350 GlcNAc C4 aminotransferase essential for protein glycosylation and virulence in  
1351 *Campylobacter jejuni*. *Journal of Biological Chemistry* **281**: 27733-27743.
- 1352 Vogel, U., Weinberger, A., Frank, R., Muller, A., Kohl, J., Atkinson, J.P., and Frosch, M.  
1353 (1997) Complement factor C3 deposition and serum resistance in isogenic capsule  
1354 and lipooligosaccharide sialic acid mutants of serogroup B *Neisseria meningitidis*.  
1355 *Infect Immun* **65**: 4022-4029.
- 1356 Wassenaar, T.M., Fry, B.N., and van der Zeijst, B.A. (1993) Genetic manipulation of  
1357 *Campylobacter*: evaluation of natural transformation and electro-transformation.  
1358 *Gene* **132**: 131-135.
- 1359 Wassenaar, T.M., Engelskirchen, M., Park, S., and Lastovica, A. (1997) Differential  
1360 uptake and killing potential of *Campylobacter jejuni* by human peripheral  
1361 monocytes/macrophages. *Med Microbiol Immunol (Berl)* **186**: 139-144.
- 1362 Wassenaar, T.M., and Blaser, M.J. (1999) Pathophysiology of *Campylobacter jejuni*  
1363 infections of humans. *Microbes Infect* **1**: 1023-1033.
- 1364 Westphal, O., and Jann, K. (1965) Bacterial lipopolysaccharide. Extraction with phenol-  
1365 water and further applications of the procedure. *Methods in Carbohydrate*  
1366 *Chemistry* **5**: 83-91.
- 1367 Whitfield, C., and Roberts, I.S. (1999) Structure, assembly and regulation of expression  
1368 of capsules in *Escherichia coli*. *Mol Microbiol* **31**: 1307-1319.
- 1369 Whitfield, C. (2006) Biosynthesis and assembly of capsular polysaccharides in  
1370 *Escherichia coli*. *Annu Rev Biochem* **75**.
- 1371 Woodman, M.E., Worth, R.G., and Wooten, R.M. (2012) Capsule influences the  
1372 deposition of critical complement C3 levels required for the killing of  
1373 *Burkholderia pseudomallei* via NADPH-oxidase induction by human neutrophils.  
1374 *PLoS One* **7**: e52276.
- 1375 Wosten, M.M., Boeve, M., Koot, M.G., van Nuenen, A.C., and van der Zeijst, B.A.  
1376 (1998) Identification of *Campylobacter jejuni* promoter sequences. *J Bacteriol*  
1377 **180**: 594-599.
- 1378 Yuki, N., Taki, T., Takahashi, M., Saito, K., Yoshino, H., Tai, T., Handa, S., and  
1379 Miyatake, T. (1994) Molecular mimicry between GQ1b ganglioside and

1380 lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Fisher's  
1381 syndrome. *Ann Neurol* **36**: 791-793.  
1382  
1383

For Peer Review

**Table 1: Sugar and NMR spectroscopy analyses of hot water / phenol extracted CPS from the wild type *C. jejuni* and its isogenic mutants.**

Monosaccharide analysis was conducted with a sugar analyzer after full acid hydrolysis. Numbers in the columns under “Sugar analysis data” represent the amounts of various sugars present, after normalization to GalN. A plus (+) or minus (-) in the NMR spectroscopy data columns represent either the presence or the absence of the specified component, respectively. GalN = galactosamine (from GalNAc), Rib = ribose, Gal = galactose (probably from the lipooligosaccharide), GlcA = glucuronic acid, Hep = heptose, Me = methyl, MeOPN = O-methyl phosphoramidate, EtN = ethanolamine, GroN = 2-amino-2-deoxyglycerol.

	Sugar analysis data					NMR spectroscopy data		
	GalN	Rib	Gal	GlcA	Hep	3,6-O-Me <sub>2</sub> on Hep	MeOPN on GalNAc	Substituent on GlcA
Wild type	1	2.6	0.1	+	+	+	-	EtN
<i>wcaG::cat</i>	1	1.2	trace	+	-	-	+	EtN, GroN
<i>mlghC::cat</i>	1	3.6	0.15	+	-	-	trace	EtN, GroN
<i>mlghB::cat</i>	1	1.4	0.18	+	-	-	+	EtN, GroN
<i>wcaG::catΔ</i>	1	0.9	trace	+	-	-	-	EtN, GroN
<i>kpsM::kan</i>	1	2.7	trace			Not analyzed		



**Table 2: <sup>1</sup>H and <sup>13</sup>C NMR data for the CPS isolated from *C. jejuni* wild type and isogenic mutants *wcaG::cat*, *mlghB::cat*, *mlghC::cat* and *wcaG::catΔ*.**

<sup>a</sup>: Data from this study.

<sup>b</sup>: The mutant *mlghC::cat* had essentially the same chemical shifts.

<sup>c</sup>: The mutant *wcaG::cat* had essentially the same chemical shifts.

<sup>d</sup>: Published data (McNally *et al.*, 2007).

<sup>e</sup>: Values without/with the MeOPN modification at position 3.

Sugar residue	Atom or position	Type	Chemical shift (ppm)	Wild type <sup>a</sup>	Mutants		NCTC 11168H <sup>d</sup>
					<i>wcaG::catΔ</i> <sub>a,b</sub>	<i>mlghB::cat</i> <sub>a,c</sub>	
Rib	1	CH	δ <sub>H</sub>	5.36	5.38	5.38	5.36
			δ <sub>C</sub>	106.3	105.9	106.0	106.1
GalNAc	1	CH	δ <sub>H</sub>	4.97	4.98	5.03	5.08/5.02 <sup>e</sup>
			δ <sub>C</sub>	106.1	106.7	106.9	105.1/104.3 <sup>e</sup>
	3	CH	δ <sub>H</sub>	4.25	4.17	4.76	4.93/4.23 <sup>e</sup>
			δ <sub>C</sub>	75.4	76.8	81.0	78.8/73.9 <sup>e</sup>
	3	MeOPN	δ <sub>H</sub>	none	none	3.77	3.77
			δ <sub>C</sub>	none	none	55.0	54.6
			δ <sub>P</sub>	none	none	13.6	not indicated
GlcA	1	CH	δ <sub>H</sub>	5.14	5.22	5.22	5.12
			δ <sub>C</sub>	99.0	99.0	99.0	98.8
Hep	1	CH	δ <sub>H</sub>	5.58	none	none	5.58
			δ <sub>C</sub>	98.3	none	none	97.9
	3	MeO	δ <sub>H</sub>	3.63	none	none	3.61
			δ <sub>C</sub>	60.8	none	none	60.7
	6	MeO	δ <sub>H</sub>	3.56	none	none	3.55
			δ <sub>C</sub>	60.8	none	none	59.3

**Table 3. Comparison of motility for wild type and mutant strains.**

The motility of the wild type and mutant strains were measured and compared quantitatively after stabbing into 0.3% agar and incubation for 48 hours under microaerobic conditions. The values shown are for the diameters of the motility halos, measured in centimeters. The mean and standard errors (SE) of three independent experiments are shown for each strain. In addition, the *p* value, as determined by one-way ANOVA, is shown. Compared to the wild type, all mutant strains were significantly reduced in motility.

<i>C. jejuni</i> strain	Diameter of halo (cm) $\pm$ SE	<i>p</i> value
wild type	3.09 $\pm$ 0.09	N/A
<i>wcaG::cat</i>	2.35 $\pm$ 0.08	< 0.01
<i>mlghC::cat</i>	2.77 $\pm$ 0.06	< 0.01
<i>mlghB::cat</i>	2.73 $\pm$ 0.06	< 0.01
<i>wcaG::cat</i> $\Delta$	2.38 $\pm$ 0.10	< 0.01
<i>kpsM::kan</i>	0.00 $\pm$ 0.00	< 0.01

**Table 4: List of primers used to construct mutants and perform qRT-PCR analyses.**

When applicable, the restriction sites are indicated in bold letters.

Primer name	Sequence (5' – 3')
Aph3 P1	GAAGATCTGATAAACCCAGCGAACCA
Aph3 P2	AGGGTCCATGGAGACATCTAAATCTAGGTAC
CatColi P2	GTCGGTACCTTATTTATTCAGCAAGTCTTG
CatColi P3	GTCATCGGGCCCTTCCTTTCCAAGTTAATTGC
<i>cj1427c</i> P2	AGGGTCCATGGGCATGTCAAAAAAAGTTTAAATTAC
<i>cj1427c</i> P3	GCGTCGGATCCTTAATTAAAATTTGCAAAGCGA
<i>cj1427c</i> P4	GTGGGTACCTAGAATGAGACTTGA
<i>cj1427c</i> P5	CTGCTAGGGCCCATATTCTGAGATAGG
<i>cj1428c</i> P2	AGGTACCATGGGCATGCAAACAAATTCAAAAATATAA
<i>cj1428c</i> P3	GCTGGATCCTCAATTTTGTGTTTTATACCA
<i>cj1428c</i> P4	GTGGGTACCTGTAGCTATCTATACGATGC
<i>cj1428c</i> P5	CTGCTAGGGCCCTCAGGATACATATACCCA
<i>cj1430c</i> P2	AGGGTCCATGGCAATAGAATTTGATATA
<i>cj1430c</i> P3	GCGTCGGATCCTTATCCTTTATTTTTAGTTGCAA
<i>cj1430c</i> P4	GTGGGTACCAAATATGGGAAATTCT
<i>cj1430c</i> P5	CTGCTAGGGCCCATTTTAAATAGGTTGG
<i>kpsM</i> P2	AGGTACCATGGTGAGTTATGATTATAGTTTATG
<i>kpsM</i> P3	GCTGGATCCTAGATTAATTAACCTTATCATTC
<i>kpsM</i> P4	GAAGATCTTGTATTTCTGTTCAATTGC
<i>kpsM</i> P6	AGGTACCATGGAGTTCTAGCAATAAATACATG
RT1425F	ACCACTTGGTACATCCGAATAGGT
RT1425R	TGCGACTATATCTTTATACATACATTG
RT1426F	TTATCGACATAAAGCATCTTGTAAAAAA
RT1426R	ATTGATAATCATCAGCAAGCTAGGAA
RT1427F	CCGCATGCACTTTATCGATCCCA
RT1427R	CTGATATTATTATTCCTCTAGCTGCT
RT1428F	AAATCACCTTGAAATAAATATTCCTCTT
RT1428R	CAAGCGGTTGCTAAATTTTTTAAAGAA
RT1430F	TTTTATAACTTATAATAAATTTCTCCCAT
RT1430R	ATTTGGACAGCTTTTACAGATGAATAT
RT1444F	TGAATCCGAATAAGTCCTTGATAAA
RT1444R	AGTGGTGGAATTCTCAAGGAAATATTT
RT1445F	AAGGTAAATCTATATGTTGCTCTTGAT
RT1445R	ATACATTGATTGCAGCACCAAGATAT
RT1447F	CCTTTATTTAGTGGTGGAAGACATTA
RT1447R	TTATCTCTAGCTGTTAAAGAACCTTG
RTKpsMF	ATTACAAAATAAATACAAAACCTCAAGTAAA
RTKpsMR	GTTAGAGAATATCATCATCAAGTTATG
RTAcCoAF	AATGTCTTGATCGTCATATGAAAACAA
RTAcCoAR	TAGCCCCAATCCTTGCACAAGCT

**FIGURE LEGENDS:**

**Fig. 1: Schematic representation of the CPS from *C. jejuni* ATCC 700819 and organization of its CPS gene cluster. Panel A:** The CPS unit comprises 4 sugars, including a 3-sugar linear backbone (sugars 1-3) that is attached to a phospholipid (PL) for anchorage in the membrane and a modified heptose (H) branch. The exact structure is also indicated and denotes additional modifications such as O-methyl phosphoramidate (MeOPN on GalNAc and heptose) and addition of ethanolamine (EtN) or 2-amino-2-deoxyglycerol (GroN) on GlcA (Karlyshev *et al.*, 2005; McNally *et al.*, 2007; St Michael *et al.*, 2002). **Panel B:** Global organization of the CPS gene cluster (Karlyshev *et al.*, 2005; Parkhill *et al.*, 2000) showing the tripartite organization of CPS genes as well as the genes relevant to this study. Diagram not to scale.

**Fig. 2: Simplified biochemical pathway highlighting the role of enzymes encoded by *wcaG*, *mlghB* and *mlghC* in the synthesis of L-glucosamine. Intricacies of this pathway were described earlier (McCallum *et al.*, 2011; McCallum *et al.*, 2012; McCallum *et al.*, 2013). This figure only highlights the linear pathway from the mannosamine precursor to the final product (additional intermediates excluded) as well as the side branch mediated by WcaG that decreases product formation via substrate scavenging. R represents OH or OMe. It is not known when methylation by *cj1426c* occurs along this pathway (as denoted by ?).**

**Fig. 3: SDS-PAGE analysis of the CPS produced by wild type and mutants. Panel A:** CPS extracted by hot water/phenol method was analyzed on a 13 cm long gel with

detection by silver staining. **Panel B:** CPS obtained by SDS solubilization of total cells and proteinase K digestion of all proteins was analysed on a 5 cm gel. Detection was performed with anti-HS:2 Penner serotyping antibody (kindly supplied by Dr M. Karmali). The *wcaG::cat* mutant is not represented on panel B as no antibody was available anymore when the proper mutant was obtained.

**Fig. 4: CPS structures of the wild type strain and the mutants.** The structures are based on the data presented in Tables 1 and 2 and in supplementary Figures S1 and S2, and also take into account previously published data for the wild type strain (McNally *et al.*, 2007; St Michael *et al.*, 2002; Szymanski *et al.*, 2003).

**Fig. 5: qRT-PCR analysis of expression of capsular genes in the wild type and mutants. Panel A:** Intra-strain comparison where the level of transcription of each gene is expressed relatively to housekeeping gene acetyl-CoA synthase in the same strain. **Panel B:** Inter-strain comparison where data from panel A are expressed relatively to wild type (Pfaffl, 2001). Cat and Kan denote the antibiotic resistance cassettes used for gene inactivation. Crosses for *wcaG::cat* $\Delta$  denote deleted genes. Additional genes deleted in this mutant are not shown as their levels of transcription were not investigated. Small arrows indicate promoters inferred from differential transcription of neighboring genes in each individual data set. The +/- refers to standard error obtained from 2 independent experiments each including triplicate. Darker and darker shades indicate more and more deviation from housekeeping levels (Panel A) or from wild type levels (Panel B), red being for reduced levels and green for higher levels. ND: none detected. NA: not

applicable, used for *kpsM* in *wcaG::catA* and *mlghC::cat* where data could not be calculated since levels were too low in the wild type reference but colors nevertheless indicate higher transcription than in wild type in both strains, with a 10-fold difference between *wcaG::catA* and *mlghC::cat* as per Panel A.

**Fig. 6: Effect of mutations on growth rates, auto-agglutination and flagellum**

**production. Panel A:** Growth curves in broth under microaerobic atmosphere determined using Klett's flasks over 24 h. Error bars represent SEM (standard error of the mean) for 3 independent experiments with 1 reading/flask at each time point. **Panel B:** Auto-agglutination quantitated by measuring the OD<sub>600nm</sub> of the top layer of a static bacterial suspension over time. Error bars represent SEM for 3 independent experiments each including 3 replicates. The same color scheme applies as in panel A. \*: p<0.01 by one-way ANOVA (with Dunnett's post test). **Panel C:** Phase contrast microscopic examination of auto-agglutination for *kpsM::kan* compared with wild type (100x magnification). **Panel D:** Electron microscopy analysis of flagella production in wild type and *kpsM::kan* strains with uranyl acetate staining.

**Fig. 7: Effect of mutations on resistance to bile salts and SDS. Panel A:** Resistance to various concentrations of bile salts. The bacteria were exposed to bile for 15 min under microaerobic atmosphere at 37°C and serially diluted for enumeration of viable cells by CFU counting. **Panel B:** Resistance to various concentrations of SDS. The cells were exposed to SDS for 15 h and cell lysis was assessed by measuring the OD<sub>600nm</sub>. The legend is the same in both panels and is shown within panel B. Error bars represent SEM

for three independent experiments, each carried out with triplicates. Data for each replica were obtained from 2 CFU spots Panel A and 1 OD<sub>600nm</sub> read for Panel B.

**Fig. 8: Effect of mutations on resistance to serum. Panel A:** Resistance to various concentrations of serum for 1.5 h under microaerobic atmosphere at 37°C. **Panel B:** Time course of resistance to 20% serum. For both panels, viability was assessed by CFU counting. The legend is the same in both panels and is shown within panel A. Error bars represent SEM. Experiments performed 3 times independently with triplicates within each experiment and determination of CFU from 2 spots for each replica.

**Fig. 9: Effect of mutations on interactions with epithelial cells.** The bacteria were centrifuged onto cell monolayers to account for motility defects. **Panel A:** Adherence to Caco-2 cells measured after 5h incubation, with elimination of unbound bacteria by washing and CFU counting of bound bacteria. **Panel B:** Invasion of Caco-2 cells measured after 5 h incubation and after elimination of externally bound bacteria via gentamicin treatment. Live intracellular bacteria were enumerated via CFU counting after lysis of epithelial cells with Triton X-100. ND: none detected. Error bars represent SEM. \*: p<0.05. \*\*: p<0.01 by one-way ANOVA. Experiments performed 3 times with duplicates within each experiment and determinations from 2 CFU spots for each replica.

**Fig. 10: Effect of the mutations on colonization and persistence in chicken intestine.** Two day old chicks were orally gavaged with 10<sup>7</sup> CFU and their caecal content in *C. jejuni* was determined after 5 days by CFU counting of plated caecal homogenate. **Panel**

**A:** Test of wild type and four mutants. **Panel B:** Test of wild type and one additional mutant. While experiments were performed following the same procedure, a slightly lower level of colonization and wider distribution of colonization levels were obtained for wild type in panel B compared with panel A, therefore, the data for the last mutant tested are displayed separately. For both panels, each data point represents an individual chick. The numbers at the top of the graphs are the number of chicks from which *C. jejuni* was isolated over the total number of chicks inoculated for each strain tested. The lower limit of detection for colonization was  $\sim 1.5$  log CFU/g. Horizontal bars represent the median of each group. Significant differences (\*\*\*\*  $p < 0.0001$ ; \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ) in colonization compared to wild type as determined by the Mann-Whitney test are indicated. A second experiment was performed with an inoculation dose of  $10^6$  and similar data were obtained (data not shown).



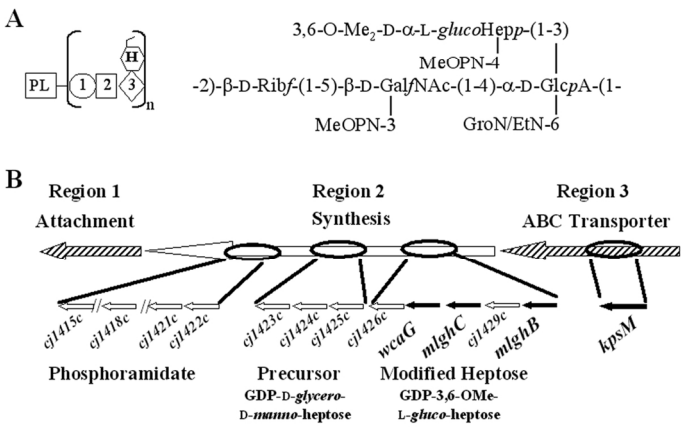


Figure 1: Wong et al.

CPS composition and CPS cluster  
149x149mm (300 x 300 DPI)



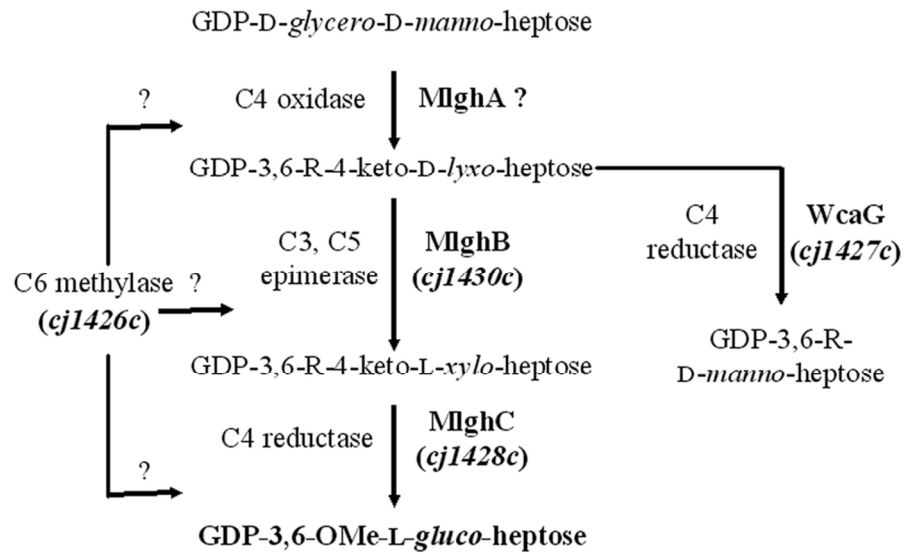
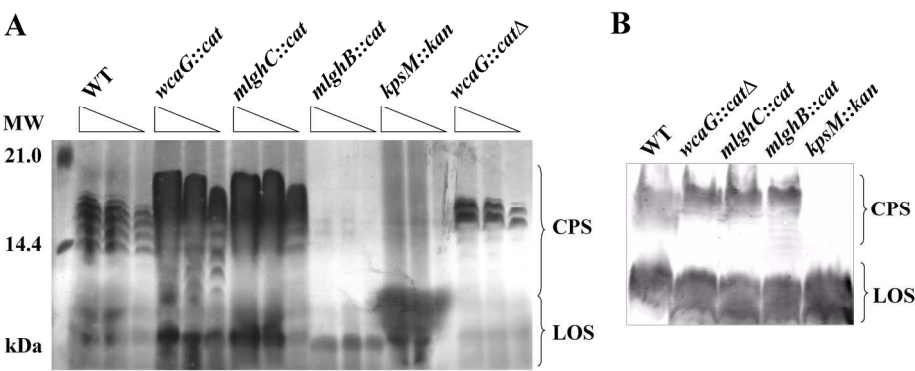


Figure 2: Wong et al.

Heptose modification pathway  
99x99mm (300 x 300 DPI)



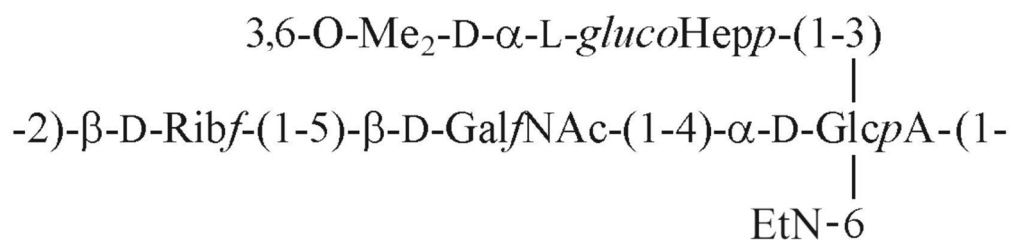
Wong et al Figure 3



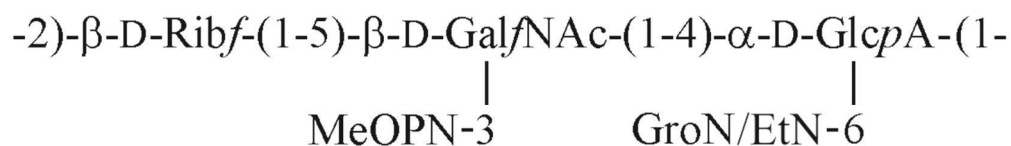
CPS gels  
199x199mm (300 x 300 DPI)



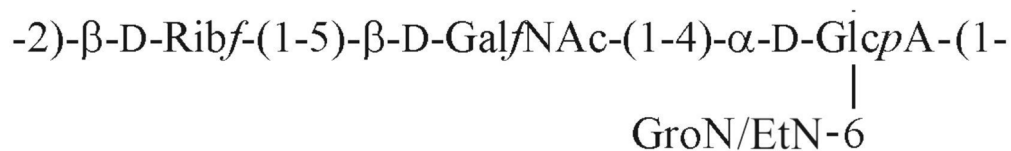
## WILD-TYPE



*wcaG::cat*, *mlghB::cat*, *mlghC::cat* (trace)



*wcaG::cat* $\Delta$ , *mlghC::cat* (major)



CPS NMR structures  
141x115mm (300 x 300 DPI)

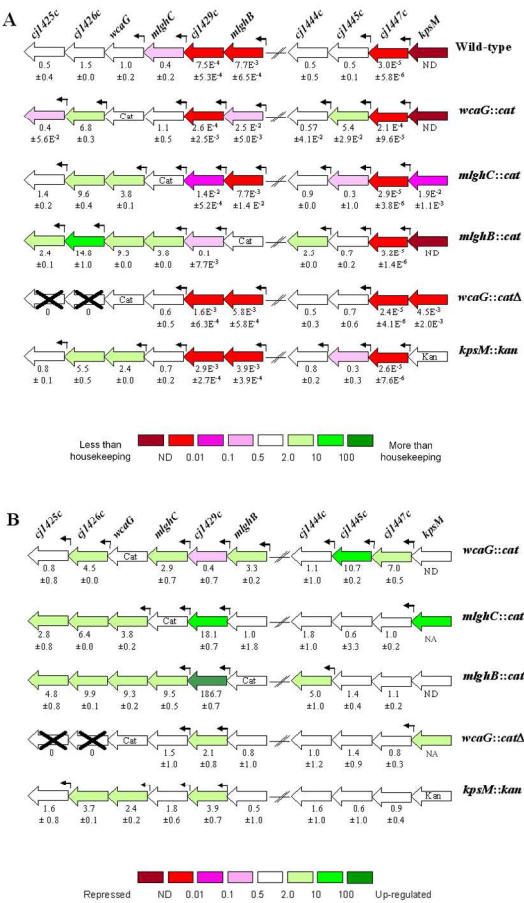
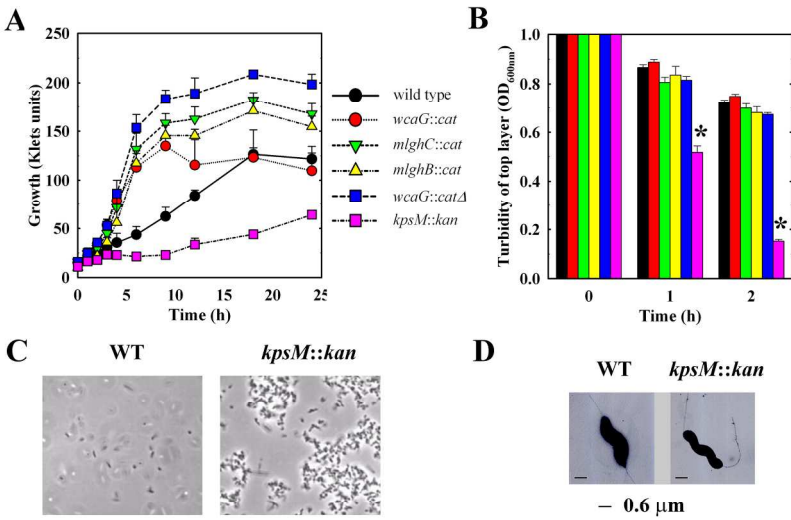


Figure 5 Wong et al

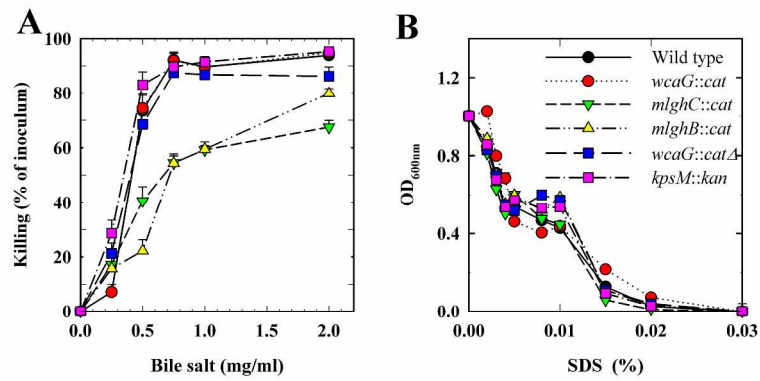
qRT-PCR analysis of CPS cluster  
240x384mm (300 x 300 DPI)

Wong et al Figure 6



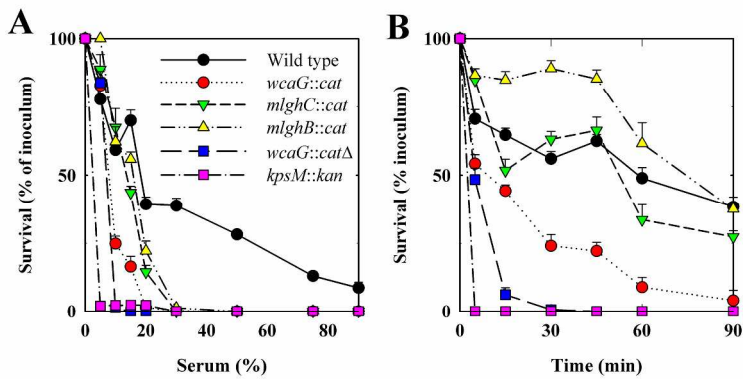
Growth, agglutination and microscopy  
199x199mm (300 x 300 DPI)

Figure 7 Wong et al



Barrier function towards bile and SDS  
279x360mm (300 x 300 DPI)

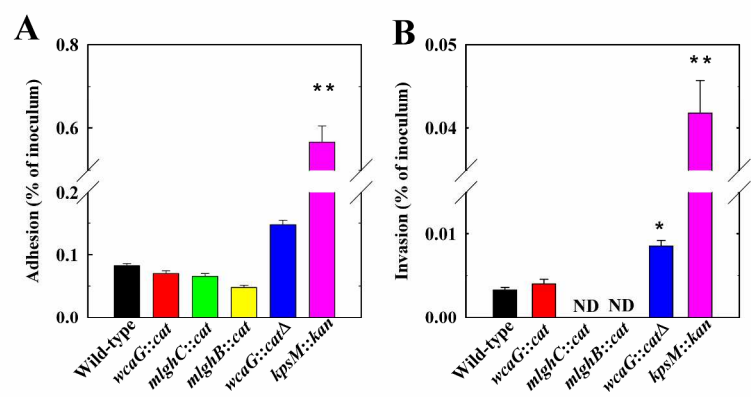
Figure 8 Wong et al



Resistance to serum  
278x360mm (300 x 300 DPI)

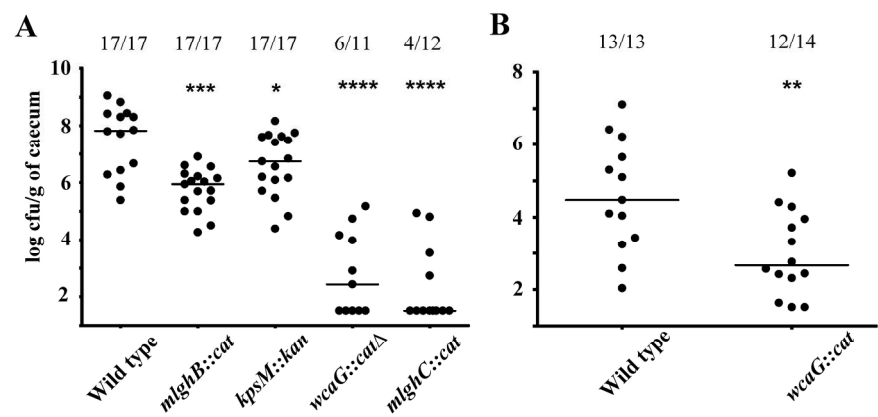


Figure 9 Wong et al



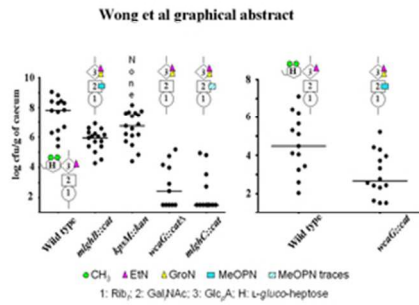
Interactions with epithelial cells  
278x360mm (300 x 300 DPI)

Wong et al Figure 10



Chicken colonization experiments  
199x199mm (300 x 300 DPI)





chicken data and CPS structure for graphical abstract  
59x39mm (300 x 300 DPI)

**Graphical abstract for Wong et al 2015****80 words.**

*Campylobacter jejuni* ATCC 700819 produces a capsule that is important for virulence and contains a modified heptose. Inactivation of any of the genes *wcaG*, *mlghB*, and *mlghC* involved in heptose modification leads to capsule lacking modified heptose and presenting altered MeOPN, EtN and NGro contents. This alters capsule barrier functions and interactions with intestinal cells and decreases colonization and/or persistence in the chicken gut. This demonstrates that these genes contribute to bacterial resistance against host defenses in the gastrointestinal tract.

**Legend for figure:**

Chicken caecum colonization by heptose modification mutants and schematic representation of their capsule composition.